Determination of biomarkers for toxicity and antiretroviral adherence in hair in South African patients

University of Cape Town

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Plagiarism Declaration

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Abstract

Background: Substance abuse is one of the many factors associated with poor levels of antiretroviral adherence and is also prevalent among HIV-infected individuals. Ethyl glucuronide, a minor metabolite of alcohol, is a stable biomarker in hair that can be used to detect and monitor alcohol consumption over long time periods. Drugs of abuse are also detected in hair. Hair provides a longer window of drug detection compared to blood and urine. Recently, hair has also been studied as an alternative matrix for adherence monitoring and concentrations of antiretrovirals in hair have been shown to be closely correlated with virologic outcomes. This study investigated the impact of substance abuse on adherence among HIV-infected patients attending an antiretroviral therapy clinic in Cape Town by measuring drug concentrations in hair. Efavirenz levels in hair were also measured to investigate the usefulness of using hair analysis as a method of adherence monitoring within the South African context.

Method: This study describes the development and validation of three liquid chromatography tandem mass spectrometry methods of hair analysis. The first method developed was for the quantification of ethyl glucuronide in 20 mg samples of hair. This method was validated over the calibration range 7.5 – 480 pg/mg. Secondly, a qualitative method was developed to screen hair samples for amphetamine, methamphetamine, cocaine, benzoylecgonine, cocaethylene and methaqualone. The final method developed was for the quantification of efavirenz in 0.2 mg samples of hair. This method was validated over the calibration range 0.625 – 40 ng/mg. The validated methods were applied to 257 samples of hair collected from 135 HIV-infected patients during visits to the clinic at weeks 16, 32 and 48. The results generated from the analysis of the hair samples were analysed in the context of additional adherence measurements collected for a related randomized controlled study.

Results: Analysis of the hair samples for ethyl glucuronide demonstrated that 27% of the samples analysed contained levels above 30 pg/mg which is the cutoff value suggested by the Society of Hair Testing to identify heavy drinkers. The results also show limitations

to using the CAGE alcohol abuse screening tool which had a poor sensitivity of only 28.8%. Eight (5.9%) out of the 135 participants were identified to be chronic drug users, and of these five (62.5%) were identified to be heavy drinkers as well. The most commonly abused drug in the screen was methaqualone. The median efavirenz levels at weeks 16, 32 and 48 were 5.52 ng/mg (IQR: 3.60 – 9.77), 5.75 ng/mg (IQR: 3.21 – 8.18) and 4.89 ng/mg (IQR: 3.10 – 7.94) respectively. Participants with the poor CYP2B6 metaboliser genotype had significantly higher median efavirenz hair concentrations compared to participants with intermediate and extensive genotypes ($P < 0.0001$). Efavirenz levels in hair and plasma samples were strongly correlated throughout the study (Spearman correlation coefficients: 0.672 – 0.741, all P values < 0.0001). Substance abuse had no impact on adherence measured by an electronic adherence monitoring device. No significant correlation was observed between adherence and levels of efavirenz in hair.

Conclusions: Methods of hair analysis were developed and successfully applied to hair samples in the context of better understanding the impact of substance abuse on adherence. The results from the analysis of the hair samples provided insight into the prevalence of substance abuse among HIV-infected patients. The strong correlation observed between levels of efavirenz in hair and plasma suggest that, in this subset of HIV-infected patients, a single plasma concentration was as good an adherence measure as a hair concentration. The hair analysis methods developed and validated in this study are novel in South Africa and demonstrate the potential of this matrix to be used in various contexts within the country.

Conference Proceedings

2016 International Association of Forensic Toxicologists (TIAFT), Brisbane Convention and Exhibition Centre, Brisbane, Australia.

Poster: Determination of Ethyl Glucuronide in hair samples of HIV-infected patients in order to better understand challenges associated with adherence to antiretroviral therapy in South African adults.

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List of Abbreviations and Units

~	approximately
%	percentage
% CV	percentage coefficient of variation
°C	degrees Celsius
µg	microgram
µl	microliter
µm	micrometre
AIDS	acquired immune deficiency syndrome
ANOVA	analysis of variance
AP	amphetamine
ART	antiretroviral therapy
ARV	antiretroviral
AU	absorbance units
AUC	area under the curve
AUDIT	Alcohol Use Disorders Identification Test
BLQ	below the lower limit of quantification
BMI	body mass index
BZE	benzoylecgonine
CDT	carbohydrate-deficient transferrin
CE	cocaethylene
CI	confidence interval
cm	centimetre
CMA	Cape Mixed Ancestry
CNS	central nervous system
COC	cocaine
cps	counts per second
Da	Dalton
DUDIT	Drug Use Disorders Identification Test
EAMD	electronic adherence monitoring device
EFV	efavirenz
EMEA	European Medicines Evaluation Agency
EtG	ethyl glucuronide
EtS	ethyl sulphate
FAEE	fatty acid ethyl esters
FDA	Food and Drug Administration
GC	Gas Chromatography
GGT	gamma-glutamyltransferase
HAART	highly active antiretroviral therapy
HCl	hydrochloric acid
HCTC	Hannan Crusaid Treatment Centre
HIV	human immunodeficiency virus
HRMS	high-resolution mass spectrometry
ISTD	internal standard
IQR	interquartile range
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography tandem Mass Spectrometry
LLE	liquid-liquid extraction

LLOQ	lower limit of quantification
LOD	limit of detection
MCV	mean corpuscular volume
Med	medium
MEMS	Medication Event Monitoring System
min	minute
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
MP	methamphetamine
MQL	methaqualone
MRM	multiple reaction monitoring
MS	mass spectrometry
ms	millisecond
$m.s^{-1}$	meters per second
MW	molecular weight
n	number of samples/repeats
NaOH	sodium hydroxide
ng	nanogram
nm	nanometre
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
PEth	phosphatidylethanol
PI	protease inhibitor
pg	pictogram
PLWHA	people living with HIV/AIDS
PrEP	pre-exposure prophylaxis
psi	pounds per square inch
QC	quality control
rpm	revolutions per minute
s	second
SAB	South African Black
S/N	signal to noise
SoHT	Society of Hair Testing
SPE	solid-phase extraction
SS	stock solution
STD	standard
STDEV	standard deviation
SWGTOX	Scientific Working Group for Forensic Toxicology
TDM	therapeutic drug monitoring
TOF	time-of-flight
UPLC	ultra performance liquid chromatography
UHPLC	ultra high performance liquid chromatography
USA	United States of America
UV	ultraviolet
V	volts
vs	versus
v/v	volume per volume
WS	working solution

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1 Literature Review

1.1 HIV and treatment in South Africa: A brief overview

Recent estimates suggest that there are over 7 million people living with human immunodeficiency virus (HIV) in South Africa. The prevalence of HIV in the adult population is 18.9%, one of the highest prevalence rates globally. In 2016 alone there were approximately 270 000 new infections. The country also has one of the largest antiretroviral therapy (ART) programs worldwide, with 56% of HIV-infected people receiving treatment in 2016 [1].

ART has advanced over the years from monotherapy to a combination of two antiretroviral (ARV) drugs to highly active antiretroviral therapy (HAART) which involves the use of three or more ARV drugs in combination. There are currently six classes of ARV drugs, namely; nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, chemokine co-receptor antagonists and integrase inhibitors. These drugs specifically target host cell receptor, reverse transcriptase, integrase and protease proteins to interfere with and prevent HIV replication [2].

Currently, the recommended first- line ART regimens for adults involve the combination of two NRTIs and either an NNRTI or an integrase inhibitor [3]. In South Africa a fixed-dose combination of efavirenz (EFV) with tenofovir and either emtricitabine or lamivudine is recommended as the preferred option for adults initiating ART [4].

Efavirenz

EFV, a NNRTI, is an important component of HAART, especially in developing countries as a result of its efficacy, low cost and low pill burden [2]. Due to its long half-life, which ranges from 52 to 76 hours in plasma following a single oral dose, it is only necessary to administer EFV once daily. The long half-life of EFV is also beneficial for adherence purposes as the relatively stable plasma concentrations allow for an occasional missed dose [5]. Shortcomings of EFV include a low genetic barrier to the development of drug-

resistant viral mutations and a high rate of central nervous system (CSN) side effects (up to 55%). Commonly experienced CNS side effects include dizziness, insomnia, impaired concentration, vivid dreams and nightmares [6, 7].

CNS side effects have been associated with high concentrations of EFV in plasma, whereas low concentrations are reported to predict treatment failure and facilitate the development of drug resistance [8]. Additionally, whilst studies have reported little intra-individual variability in EFV plasma levels, high inter-individual variability among patients has been demonstrated [8, 9]. EFV is metabolised primarily by the enzyme CYP2B6, which is known to show considerable inter-individual variability [10]. Therefore, the high variability in EFV plasma concentrations among patients is most likely due to CYP2B6 genetic polymorphisms that may result in increased or reduced enzyme activity, subsequently influencing the metabolism of EFV [11].

Individuals with increased enzyme activity have been reported to have sub-therapeutic EFV concentrations [12] whereas reduced enzyme activity, causing slow metabolism of EFV, has been associated with elevated EFV plasma concentrations as well as an increase in CNS side effects [13, 14]. The prevalence of the slow metaboliser polymorphism differs among populations and is significantly higher among sub-Saharan Africans, putting this population at risk of significantly elevated EFV plasma concentrations as well as susceptibility to toxic CNS side effects that often lead to poor adherence [6, 11].

1.2 Adherence to Antiretroviral Therapy

Long-term adherence to ART is essential for the successful treatment of HIV. Poor adherence can result in treatment failure as well as drug resistance [15]. Whilst adherence rates of ≥ 95% are generally needed to maintain virologic suppression, actual adherence rates are often much lower [16]. A meta-analysis of 84 studies conducted across 20 countries found that only 62% of patients reported adherence of ≥ 90% [17]. Interestingly, better adherence was reported in resource-poor settings. Nonetheless, adherence rates in sub-Saharan Africa are often poor and decrease over time [18].

Measuring levels of adherence among patients is critical in monitoring treatment outcomes and identifying patients that are in need of interventions. However, accurately measuring ART adherence is challenging and further complicated by the many different methods available for measuring adherence [19].

1.2.1 Methods of measuring adherence

Commonly used adherence measures include both indirect and direct methods of measurement. Indirect measures of adherence include patient self-reports, pill counts, pharmacy refill records and electronic drug monitoring. Monitoring levels of drugs or drug metabolites in biological matrices, usually plasma, known as therapeutic drug monitoring (TDM) is an example of a direct method of measuring adherence. There are advantages and disadvantages to each of these methods, which have been previously reviewed [16, 18–21], and no one method is considered to be the gold standard [15]. However, using a combination of adherence measures might provide a more accurate result [21]. Duong et al. [22] suggests using an approach based on both self-reported adherence and the measurement of ARV levels in plasma as complimentary measures of monitoring adherence.

Self-report is currently the most widely used adherence measure and generally involves asking patients about the number of doses of ART they have taken or missed over a short period of time. Whilst this method of measuring adherence is simple and inexpensive, it is also subjective and usually overestimates adherence due to recall bias and the social desire to please [18]. TDM on the other hand is an objective measure of medication adherence that is used in a variety of clinical settings. However, using TDM as a routine approach for monitoring adherence to ART has been debated [23, 24]. TDM is limited by cases of intra-individual variability in plasma ARV levels among patients receiving standard doses [25]. Additionally, plasma concentrations are susceptible to 'white coat effects' where adherence improves just prior to clinic visits [26] and only provide a 'snapshot' of recent adherence. More recently, hair has been investigated as a matrix for monitoring long-term adherence to ART [27], and has the potential to

overcome some of the limitations associated with measuring ARV levels in plasma. The use of hair analysis in monitoring adherence to ART is discussed in more detail in Section 1.6 of this review.

1.2.2 Factors affecting adherence - the impact of substance abuse

Many factors have been associated with poor adherence including forgetfulness, being away from home, conflicts with daily routine, frequency and severity of side effects, social influences, depression and other psychiatric disorders and active substance abuse [16, 28]. At a rural health centre in South Africa, 168 HIV-infected patients receiving ARV medication were interviewed using pre-structured questionnaires to determine factors associated with poor adherence within a rural setting [29]. Results of the study indicated that poor adherence was seen in patients who were unemployed, unmarried and consuming alcohol.

One of the most frequently studied factors of poor adherence to ART is substance abuse [30]. Substance abuse is prevalent among HIV-infected individuals and is not only associated with poor adherence, but also with decreased utilisation of ART and virologic suppression [31]. Whilst both drug and alcohol use have been shown to impact adherence [31, 32], more recent studies have focussed on the impact of alcohol and drinking behaviour on adherence to ART [33, 34]. A combined analysis of 40 studies demonstrated that the risk of non-adherence was almost double among HIV-infected individuals who drank alcohol compared to those who did not drink or drank relatively less alcohol [34]. Moreover, this effect was more evident for heavy drinking compared to social drinking.

Alcohol consumption not only impacts adherence to ART, but also has the potential of increasing the acquisition and transmission of HIV, predominantly through riskier sexual behaviour. In addition, the negative impact of alcohol on the immune system may result in an increased susceptibility to HIV infection, and once infected accelerate HIV disease progression [35–37]. Alcohol consumption therefore plays a role at various points within

the process of HIV infection, and in light of this, alcohol has been described to play a major role in the HIV epidemic in sub-Saharan Africa where there are high levels of both HIV infection and heavy drinking [38].

Substance abuse and antiretroviral adherence in South Africa

Alcohol is the most commonly abused substance in South Africa with heavy episodic drinking (drinking more than 60 g or more of pure alcohol in one occasion) being prevalent among South Africans [39]. In two recent studies the prevalence of heavy drinking and problematic drug use among HIV-infected individuals attending clinics in South Africa were reported to be 37% and 13% [40] respectively, and 46% and 15% [41] respectively. Although, previous studies conducted in sub-Saharan Africa have reported lower prevalence rates of heavy drinking among HIV-infected individuals, ranging from 7% to 31% [42]. However, different self-report approaches used in these studies to measure alcohol and drug use makes it difficult to compare different prevalent rates reported.

Several studies have investigated the impact of alcohol and/or drug use on ART adherence and/or disease progression among HIV-infected individuals in South Africa [40, 41, 43–47]. Similar findings were reported in these studies demonstrating a negative relationship between alcohol and/or drug use and non-adherence. Moreover, heavy drinking and/or drug use has also been reported to be predictive of skipping or stopping ARVs associated with a decrease in CD4 counts, an increase in HIV disease progression and poorer health outcomes [41].

Both alcohol use and abuse have been associated with poor adherence [45, 47]. Kekwaletswe and Morojele [47] found a significant association between the level of alcohol use and degree of adherence, in which the lowest levels of adherence were associated with higher levels of alcohol consumption. A study based on daily phone interviews with HIV-infected patients about the number of alcoholic drinks consumed and missed ARVs found that when patients drank they were at an increased likelihood of not taking their ARVs, supporting the hypothesis of an event-level relationship

between alcohol and non-adherence [46]. Interestingly, three patterns of ART use among alcohol drinkers have been identified: (1) taking ART earlier than required when planning to drink (2) taking ART while drinking alcohol and (3) skipping ART doses when drinking alcohol [48].

Even though several studies within South Africa have reported the negative impact that alcohol and/or drug use has on HIV treatment, there have been few attempts to deliver interventions focused on reducing alcohol consumption and drug use among HIV-infected individuals [47, 49–51]. One intervention study, focussed on reducing alcohol and drug use among women living with HIV, was effective in helping women to stop using alcohol, and to a lesser extent drugs, when assessed at a 12 month follow up [51]. The brief intervention consisted of four one hour intervention modules that were delivered over two contact sessions. Topics covered in the contact sessions included alcohol and drug use, sex risk behaviours, violence and gender inequality. Myers et al. [49] further investigated the acceptability of a brief alcohol focused intervention for people living with HIV. Findings suggested that the brief intervention consisting of four sessions held over two weeks was acceptable to and appropriate for heavy drinkers receiving ART. Further research is required to investigate the best way in which to implement interventions focused on reducing alcohol and drug use in HIV clinics.

A limitation of the studies discussed above is that, where relevant, alcohol and/or drug use were measured using self-report measures, such as the Alcohol Use Disorders Identification Test (AUDIT) [52] and/or the Drug Use Disorders Identification Test (DUDIT) [53], and the results are therefore subject to recall bias. Objective measures of alcohol and drug use are needed in order to accurately measure substance use. The analysis of hair for biomarkers and drugs, an example of an objective measure, will be discussed in detail for the remainder of this review.

1.3 An introduction to hair analysis

In 1979 the first report on hair analysis was published by Baumgartner et al. [54]. Using radioimmunoassay, morphine was detected in the hair of heroin abusers. Furthermore, differences in the concentration of morphine along the hair shaft were observed that correlated to the time of drug use. This initial discovery was followed by many other studies on the analysis of hair, mainly for drugs of abuse.

Unlike with other biological matrices, such as blood and urine, drugs remain in hair and can be detected long after the drugs have been eliminated from the rest of the body. As a result, hair analysis provides a longer window of drug detection (months to years). The main advantage of hair analysis is its ability to provide a historical profile of an individual's exposure to drugs following chronic as well as once-off drug use [55]. Other advantages of hair analysis include; a simple and non-invasive sample collection, hair samples are easy to store and transport and cannot be easily tampered with. In addition, drugs incorporated into hair are stable for long periods of time [56].

Hair analysis is currently a well-established complimentary technique that is used routinely for the detection of drugs with a range of applications in both forensic and clinical toxicology [56]. Despite the increase in the number of laboratories offering hair testing there are no recognized standardized methodologies that exist. As a result the Society of Hair Testing (SoHT) have published guidelines in order to assist laboratories with hair analysis [56]. These guidelines include recommendations for sample collection and preparation as well as testing procedures. One of the main challenges associated with hair analysis is the correct interpretation of results. In order to fully understand and interpret hair analysis results, it is important to have a basic understanding of the anatomy and physiology of hair as well as the processes whereby which drugs are incorporated into hair and what factors possibly influence these processes [55].

1.3.1 Hair anatomy and physiology

Hair is a complex matrix typically made up of cylindrical shafts. Each cylindrical shaft is made up of tightly compacted cells that grow from follicles. The cuticle forms the outer protective layer and is mostly composed of sulphur-rich proteins, known as keratin. This layer is often damaged by chemicals, heat or light. The interior structure of the hair shaft is made up of cortical cells which contain a variety of chemicals, including melanin. Melanin is the principal pigment of hair and the colour of hair varies depending on the amount, distribution and type of melanin present [57].

Hair follicles are located in the epidermis and surrounded by dense capillary networks. Three types of glands are associated with hair follicles, namely the sebaceous, apocrine, and sweat glands, depending on the site of the hair. Sweat and sebaceous glands are located over almost the entire surface of the body, whilst apocrine glands are localized in the eyelids, external auditory canal, axilla (underarm) and perineal region. The sebaceous and apocrine glands secrete directly into the hair follicle, whereas the sweat glands secrete near the exit of the hair follicle [58]. The secretions of these glands cover the hair shaft and therefore make it possible for drugs to be transferred into hair through these secretions [57].

Hair does not grow continuously, but rather in a cycle of three phases. The first, the anagen phase, is a phase of increased metabolic activity and active growth during which nutrients are delivered to the hair follicle by the surrounding capillary network [57]. Additionally, extraneous substances present in the blood, such as trace metals or drugs are thought to be incorporated into the hair during this phase [59]. It is estimated that the anagen phase lasts between 4 – 6 years depending on the type of hair. The second and third phases of the hair growth cycle are the catagen (transition) and telogen (resting) phases, which last a few weeks and 4 – 6 months respectively. On average, 85% of hair on the human head is in the anagen phase and the remaining 15% is in the telogen phase during which no hair growth occurs [57].

As not all hair grows at one specific time, it is difficult to determine the exact amount that hair grows in any given period of time. It is usually assumed that human head hair grows at an average rate of 1 cm/month, although reported growth rates range from 0.6 – 3.36 cm/month. The most important factors that determine the rate of hair growth are the type and the location of the hair. For example, head hair grows more quickly than pubic or axillary hair whilst beard hair grows the slowest. Other factors that further affect the rate of growth are race, sex and age [57].

1.3.2 Drug incorporation into hair

The exact mechanisms whereby which drugs are incorporated into hair are not yet fully understood, however it is generally accepted that there are three main routes through which drugs can be incorporated into hair, as illustrated in Figure 1.1. The first, and probably most important route, is through active or passive diffusion from the bloodstream during hair growth. Secondly, drugs may be incorporated through diffusion from sweat and sebum that cover the growing hair follicle and surface of the hair shaft, and thirdly from the external environment after the hair has formed and emerged from the skin [60]. It is likely that each of these routes contributes to drug incorporation, but the extent of the role that each route plays is still not clear and may vary greatly depending on the individual as well as the drug [61].

Various factors are known to affect drug incorporation into hair. Pragst et al. [62] describes three main factors that influence the process, namely; the lipophilicity and basicity of the substance as well as the melanin content of hair. Lipophilic molecules easily penetrate membranes and diffuse across concentration gradients. During metabolism the hydrophilicity of drugs is increased and as a result polar metabolites are incorporated into hair to a lesser extent compared to their lipophilic precursors.

The intracellular pH of keratinocytes and melanocytes (pH 3 – 6) is more acidic than plasma (pH 7.3) and under these conditions basic drugs are incorporated into hair to a

greater extent than neutral or acidic drugs [63]. Studies have demonstrated that basic drugs also have a greater binding affinity to melanin and are therefore present in higher concentrations in pigmented (darker) hair [64–67]. As a result of the acidic environment and the significant binding affinity to melanin, basic drugs tend to accumulate in hair, especially darker hair, at much higher concentrations compared to acidic and neutral drugs [62].

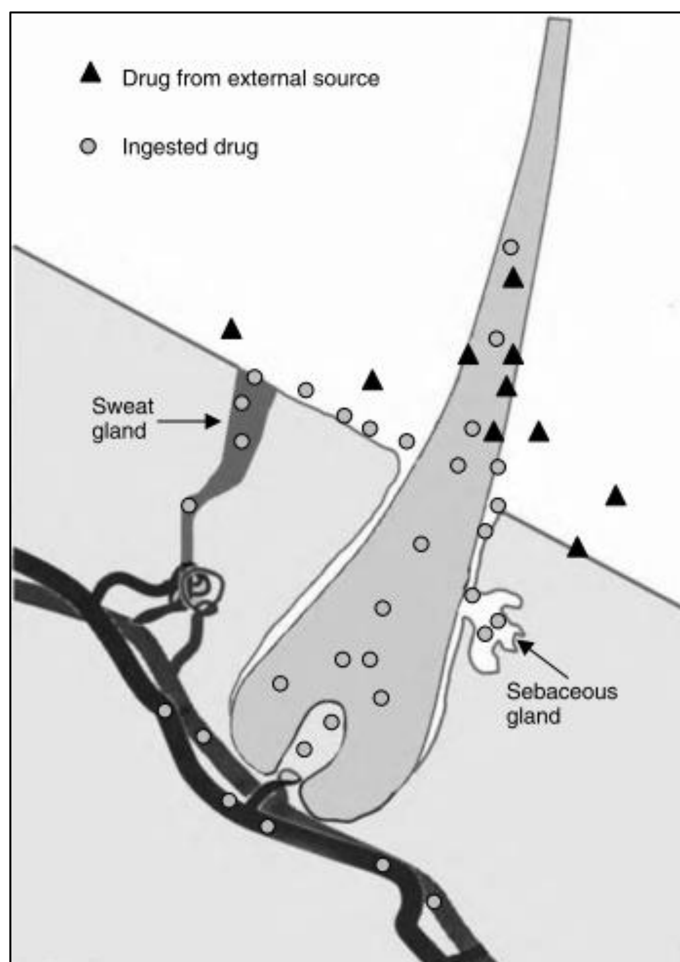

Figure 1.1 Three routes of drug incorporation into hair: ingested drugs can enter the hair from the (1) bloodstream as well as (2) through sweat and sebum, and drugs from the (3) external environment can also be incorporated into hair. Copyright © 2015 , Elsevier, Cooper [63].

1.3.3 Analytical techniques in hair analysis

Hair analysis generally consists of a number of steps, as illustrated in Figure 1.2. A brief description of each of the steps is provided below and more detailed discussions will follow for the drugs of interest relevant to the study in Sections 1.4, 1.5 and 1.6 of the literature review.

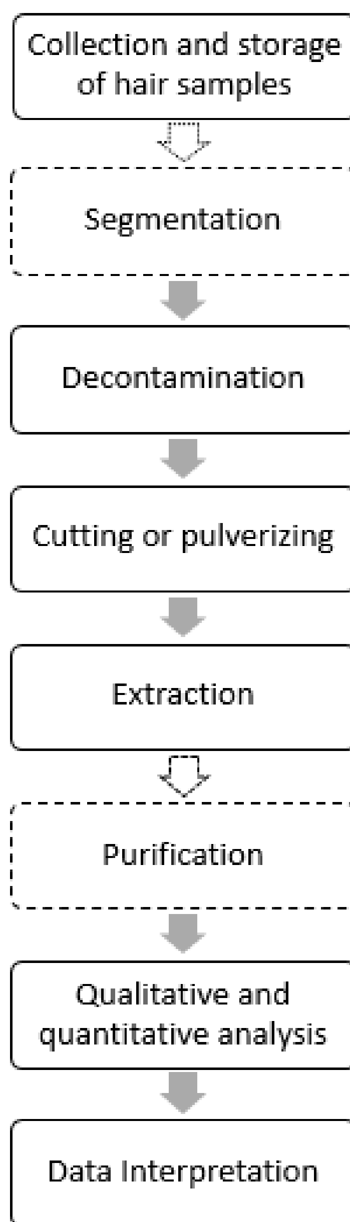

Figure 1.2 Steps involved for the analysis of hair (dotted lines represent optional steps).

1.3.3.1 Sample collection and preparation

Collection and storage of hair samples

Head hair samples are the easiest to collect and the preferred hair sample for drug analysis. The SoHT recommends that hair samples are cut as close to the scalp as possible from the posterior vertex region of the scalp [56] as this is where the growth rate is the most constant [57]. It is important to consider that it takes approximately 7 – 10 days for growing hair to exit the surface of the scalp and therefore hair cut from the scalp does not represent the most recent hair growth [55]. Usually about a pencil thickness or a 'lock of hair' is required for hair testing. In the absence of head hair, or if the hair is too short to be collected, hair samples can be collected from alternative sites including pubic, axillary and beard hair [56].

The recommended procedure for storing hair samples is to wrap collected samples of hair in aluminium foil before placing in a paper envelope. Head hair samples should be aligned and the root end of the sample clearly marked. Hair samples must be stored in a dry and dark environment at room temperature after collection. To assist with the interpretation of the results it is important to record all relevant information including the site from which the hair sample was collected, the length and colour of the hair as well as the use of any cosmetic treatments [56].

Segmentation

By cutting hair samples into segments and analysing each segment of hair separately, a more detailed profile of an individual's exposure to drugs can be obtained [55]. Measured segments between 1 and 3 cm are generally used. Segmental analysis can only be performed using head hair and in cases where the root end of the hair can be clearly identified [56].

Decontamination by washing

Hair samples are washed prior to analysis to remove any hair care products, sweat or sebum that might interfere with the analytical procedures. Additionally, hair samples are also washed to remove any external contamination of drugs from the environment

that could lead to false positive results. To date, there is no general consensus regarding the wash procedure. Although, the SoHT does recommend that wash procedures include both organic solvents and aqueous solutions [56]. The use of organic solvents, such as dichloromethane, are more advantageous as they only remove surface contamination from hair compared to aqueous solutions and methanol that cause hair to swell resulting in drugs incorporated into the hair matrix to be extracted. The ideal wash procedure should remove all surface contaminants from hair without causing drugs incorporated into hair to be extracted [62].

Extraction

Once hair samples have been washed and dried they are usually cut into smaller pieces (2 – 3 mm) or pulverized into a fine powder. This increases the surface area of the hair that is in contact with the extraction solvent and thereby increases the potential for drug extraction [68]. In order for drugs present in hair to be detected and quantified, they must first be released from within the hair matrix. Different approaches have been proposed in order to do so. One approach is to incubate hair in solutions that do not damage the hair such as methanol or buffered solutions. This approach is often facilitated by ultrasonication or pulverization to disrupt the hair structure. Another approach is to incubate hair in alkaline solutions that cause complete digestion of the hair [69]. The choice of extraction procedure depends on the chemical properties of the analytes that are to be extracted. Using unsuitable extraction procedures that are not targeted to specific drugs can significantly compromise the sensitivity of the method. Depending on the extraction method the resulting extract can either be analysed directly, or require further purification/clean-up using either liquid-liquid extraction (LLE) or solid-phase extraction (SPE) [56].

1.3.3.2 Qualitative and quantitative analysis

Chromatographic techniques coupled with mass spectrometry, such as LC-MS and GC-MS, are used for the detection and quantification of drugs and metabolites in hair. Drug levels in hair are considerably lower than those observed in matrices such as blood and

urine and often require methods with increased sensitivity. Therefore, methods incorporating tandem mass spectrometry are increasingly used in hair analysis [55].

1.4 Hair analysis for ethyl glucuronide as a measure of alcohol consumption

1.4.1 Alcohol markers

Unlike self-report questionnaires biomarkers are able to measure alcohol intake without the limitation of recall bias. Alcohol intake is routinely monitored by both indirect and direct markers. Indirect markers include measures of gamma-glutamyltransferase (GGT), mean corpuscular volume (MCV) and carbohydrate-deficient transferrin (CDT) in samples of plasma and serum [70]. However, the sensitivity and specificity of these markers for the detection of alcohol intake is poor. Furthermore, these markers are influenced by various factors such as gender, age, body mass index and non-alcohol related illnesses [71].

Direct markers of alcohol intake include ethanol as well as the direct products of ethanol metabolism. About 92 – 95% of ethanol consumed is removed from the body by oxidative metabolism. A small proportion of ethanol undergoes non-oxidative metabolism to form ethyl glucuronide (EtG), ethyl sulfate (EtS), fatty acid ethyl esters (FAEE) and phosphatidylethanol (PEth) (Figure 1.3) [72]. These markers are only present when alcohol has been consumed making their detection highly sensitive and specific in measuring alcohol intake [73]. Direct markers can be assessed in blood and urine, however, the window of detection is narrow (hours, days or weeks). Hair, on the other hand, provides a much longer window of detection (from months to years) and concentrations of EtG and FAEE in hair are used to assess drinking behaviour over longer time periods [74].

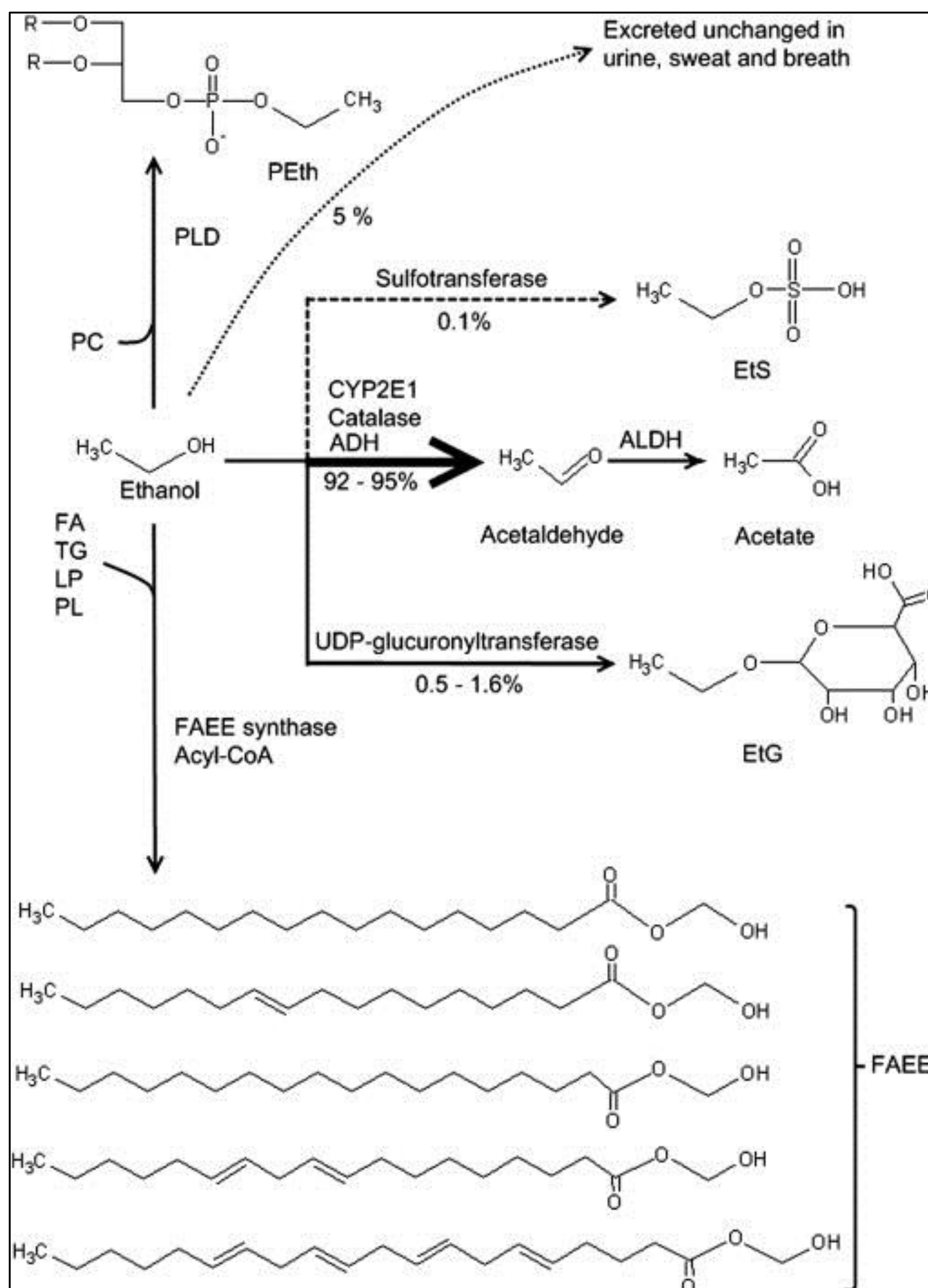

Figure 1.3 The metabolism of ethanol and the formation of non-oxidative ethanol metabolites. FA, fatty acids; TG, triglycerides; LP, lipoproteins; PL, phospholipids; PC, phosphatidylcholine; PLD, phospholipase D; CYP2E1, cytochrome P450 2E1; ADH, alcohol dehydrogenase; ALDH, acetaldehyde dehydrogenase; UDP, uridine diphosphate. Copyright © 2013 Elsevier, Maenhout et al. [72].

FAEE are formed after alcohol consumption by an enzyme-mediated esterification of ethanol with free fatty acids, triglycerides, lipoproteins and phospholipids (Figure 1.3) [72]. Whilst there are more than 20 different FAEE, the SoHT recommends that four specific FAEE are measured in hair for the assessment of alcohol intake, namely ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate [75]. Although, the most recent consensus reached by the SoHT recommends that for the interpretation of results, only the concentration of ethyl palmitate should be used [76].

Studies have shown that the regular use of ethanol containing products such as hair lotions, hair sprays and deodorants can lead to FAEE concentrations in the range typical for alcohol abuse [77, 78]. It is also possible that endogenous levels of FAEE can be found in hair without any exposure to alcohol [79]. Therefore, there is a risk of false positive results when analysing samples of hair for FAEE alone. As a result, the SoHT has proposed that the analysis of EtG in hair should be the first choice when assessing for alcohol abstinence [75]. When assessing for heavy drinking, EtG and FAEE can be measured alone or in combination. Whilst studies have shown that combined use of both markers increases the accuracy of results [78, 80], the interpretation of results can become complicated when the results from each of the markers differ. In order to assist with interpreting combined results Pragst et al. [81] has proposed a scheme for the combined interpretation of FAEE and EtG in hair. However, EtG alone can be used to assess both alcohol abstinence and abuse. The rest of this section will focus on EtG in hair as a biomarker for alcohol consumption.

1.4.2 Ethyl glucuronide

EtG is formed from the phase II conjugation reaction of a small proportion of ethanol (0.6 – 1.5%) with glucuronic acid by UDP-glucurontransferase [82] (Figure 1.3). Of the total amount of ethanol that is ingested, less than 0.06% is eliminated as EtG in the urine [83]. EtG is only produced following alcohol consumption and is a stable, non-volatile and water-soluble metabolite that can be detected in the body long after the complete

elimination of alcohol [84–87]. Moreover, since EtG is also incorporated into hair, it has been established as a valuable biomarker to detect and quantify alcohol consumption over long periods of time [74].

1.4.2.1 Incorporation of ethyl glucuronide into hair

EtG, a hydrophilic molecule with acidic properties (pKa = 3.21), is deprotonated at physiological pH and does not bind to melanin in the hair. Consequently the incorporation rate of EtG from blood to hair has been estimated to be low, and it was initially thought that the main route of incorporation was through the deposition of EtG from sweat [80]. Kharbouche et al. [88] administered ethanol by intragastric route to rats in order to investigate the influence of ethanol dose on the incorporation of EtG in rat hair. The results indicated that EtG was incorporated into hair in a dose-dependent manner and further that concentrations of EtG in hair tended to be proportional to the concentrations of EtG in blood. Since rats do not sweat the results from the study suggest that the most likely route for EtG incorporation is actually from the bloodstream and that EtG present in blood is incorporated into hair during hair growth.

This result was confirmed by a study investigating the concentration of EtG in daily shaved beard hair after single high alcohol doses [89]. Three human volunteers drank a single dose of between 153 and 200 g of ethanol over 5.5 hours. Daily beard hair shavings were collected each morning for a period of 32 days. Concentrations of EtG were detected approximately 14 hours after the start of drinking and the highest concentrations were detected on days 2 – 4. The EtG concentration gradually decreased to the lower limit of quantification (LLOQ) (2 pg/mg) on days 8 – 10. By arranging the shavings for each volunteer in the approximate position in the root hair at the time of alcohol consumption it was concluded that most of the EtG had to have been incorporated by diffusion from blood and that deposition from sweat did not play a major role. Although, the authors do suggest that sweat could play a more important role in the incorporation of EtG in longer hair where the length of the hair provides a larger area for deposition from sweat compared to the beard stubble left after shaving.

The incorporation of EtG in hair is not a simple process and involves many steps that are influenced by properties of the individual as well as external factors. Based on the work by Nakahara et al. [90] it has been proposed that the amount of EtG incorporated in hair is best correlated with the area under the curve of ethanol (AUC_{EtOH}) in plasma [62]. Due to the zero-order metabolism of ethanol the AUC_{EtOH} depends not only on the alcohol dose, but also on the drinking pattern [81]. For example, Pragst et al. [81] demonstrated that drinking 120 g of ethanol as a single dose within three hours compared to drinking 120 g in six doses of 20 g every 2.5 hours resulted in a 46-fold increase of AUC_{EtOH}. Since the concentration of EtG in hair is determined essentially by the AUC_{EtOH}, it follows that the concentration in hair is also influenced by the drinking pattern (for example drinking small amounts of alcohol every day or binge drinking the same amount over the weekend), with an increased sensitivity for binge drinking.

1.4.2.2 Techniques for the determination of ethyl glucuronide in hair

Due to the poor incorporation rate of EtG in hair, EtG is only detected in hair in very small amounts (in the range of picogram per milligram of hair), and requires the use of sensitive analytical methods. The first methods to be published for the determination of EtG in hair were in 2000 by Skopp et al.[91] and Alt et al. [92] using GC-MS techniques, and in 2002 the first LC-MS/MS method was published [93]. In recent years a number of methods have been developed and validated, with the majority of methods based on LC-MS/MS techniques [74]. GC-MS techniques require that substances are sufficiently volatile and stable at high temperatures and it is often the case that hair extracts must be derivatized before GC-MS analysis. Unlike in GC-MS, LC-MS/MS techniques do not require a derivatization step and therefore complications involving volatility, stability and derivatization issues are avoided [62].

To standardise procedures and the interpretation of results, the SoHT has published a consensus regarding hair analysis for the assessment of abstinence and chronic excessive alcohol consumption [75]. To distinguish between abstinence and social (non-risk) drinking a cutoff of 7 pg/mg has been proposed and a concentration of EtG ≥ 7

pg/mg in head hair is strongly suggestive of repeated alcohol consumption. Chronic excessive alcohol consumption (heavy drinking or alcohol abuse) is characterised by concentrations of EtG ≥ 30 pg/mg in head hair. The SoHT recommends in the consensus that results are to be interpreted with caution if hair samples less than 3 cm or greater than 6 cm are used. This section provides an overview of the various sample preparation methods as well as LC-MS/MS methods that have been published for the determination of EtG in hair.

Sample preparation

In most cases a sample size of about 30 – 50 mg of hair is used to measure EtG levels [74]. Even though it is unlikely for hair samples to become contaminated by EtG, washing procedures are still included in sample preparation methods, mainly to remove impurities that could interfere with the analysis [94]. Most wash procedures incorporate a number of sequential washing steps using solvents of different polarity. There is no consensus regarding the washing procedure to be used, although sequential washing with dichloromethane followed by a short methanol rinse has been used in a number of methods [81, 95–99]. In order to prevent extraction of EtG during this step it is preferable to use solvents that do no cause the hair to swell such as dichloromethane and acetone [94]. Even so, in many instances, water has been used as a wash solvent [78, 100–104], which is surprising if the polar nature of EtG is considered.

After washing, dry hair samples are either cut into short pieces or pulverized/ground into a fine powder. The SoHT recommends that hair samples are pulverized prior to the extraction of EtG [75]. Pulverizing hair into a fine powder increases the surface area from which EtG can be extracted, resulting in a significant improvement in the extraction efficiency. This significant increase in the amount of EtG extracted from powdered hair compared to cut hair was first demonstrated by Albermann et al. [105] and later by Mönch et al. [106] and Kummer et al. [107]. The impact of different grinding procedures on the structure of the hair was illustrated by Kummer et al. [107] using electron microscopy. As shown in Figure 1.4, extensive pulverization of the hair completely destroyed the structure of the hair thereby increasing the surface area.

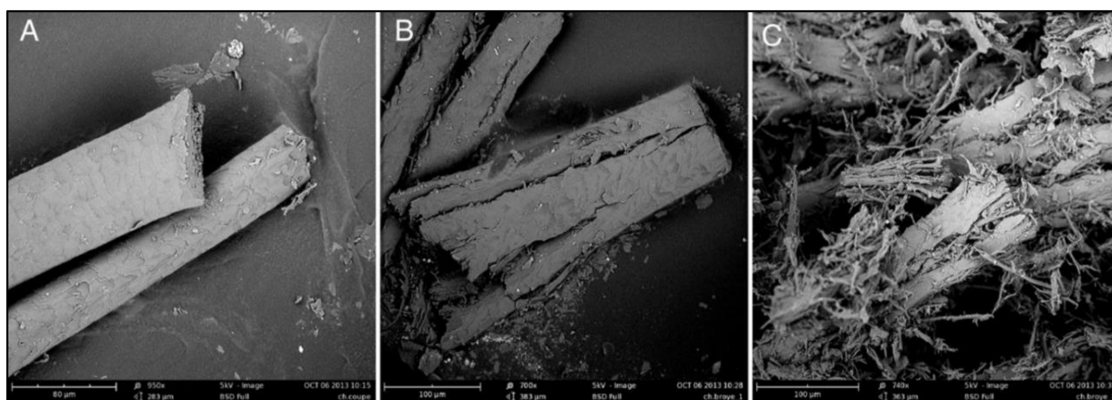

Figure 1.4 Microscope images of hair cut into small pieces (A), weakly pulverized (B), and extensively pulverized (C). Weakly pulverized hair was subjected to one grinding cycle of 30 s at a speed of 6500 rpm and for extensively pulverized hair three cycles of 60 s at a speed of 6500 rpm and a cooling time of 2 min in between each cycle was used. Copyright © 2014, Oxford University Press, Kummer et al. [107].

A study comparing the extraction kinetics of EtG from cut hair compared to powdered hair showed that the extraction time could be significantly decreased when using powdered hair [97]. The extraction of EtG from the powdered hair reached a plateau after 4 hours, whereas after 18 hours the extraction of EtG from the cut hair was not yet complete. Using micropulverized extraction, a combination of simultaneous pulverizing and extraction, quantitative extraction of EtG was obtained within 30 min [108]. Water is the best solvent for extracting EtG from hair [109] and has been applied in most extraction procedures along with ultrasonication (1 – 4 hours) followed by overnight incubation [74]. Following extraction, a sample clean-up step is often performed to remove impurities and thereby increase the sensitivity of the procedure. Most clean-up steps are based on SPE techniques [74]. Several methods have been published, however, that have obtained adequate sensitivity without incorporating a clean-up step [95, 99, 105, 110].

Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS)

Janda et al. [93] published the first LC-MS/MS method for the determination of EtG in hair in 2002 with an LLOQ of 102 pg/mg. The first fully validated method was published by Morini et al. [99] in 2006 and could measure EtG concentrations as low as 3 pg/mg.

Both of these methods, including a method published by Albermann et al. [95] (LLOQ = 4 pg/mg), required post-column addition of acetonitrile to enhance analyte ionization. As mentioned previously, EtG is a highly polar molecule and therefore for retention on reversed phase LC columns a highly aqueous mobile phase is needed. Under these conditions ionization in the electrospray ionization mode is low leading to sensitivity issues [74]. The sensitivity problem has also been solved by using hydrophilic interaction liquid chromatography (HILIC) columns with a high organic content in the mobile phase [111–113].

Most of the LC-MS/MS methods published, including a recently published method by Oppolzer et al. [114] in 2016, use gradient chromatography with mobile phases made up of formic acid and acetonitrile. Generally the transitions 221 → 75 as the quantifying ion and 221 → 85 as the qualifying ion have been used to monitor EtG [79] and in all recent methods the published LLOQ has been in the low pg/mg range [74].

1.4.2.3 Data Interpretation

When reporting hair analysis results for EtG it is important that results have been interpreted with caution and that factors that might influence results have been carefully considered. The type of hair sample as well as individual factors can influence the incorporation of EtG in hair resulting in variability between observed EtG concentrations [74]. A few of these factors are discussed below.

Effect of hair colour and cosmetic treatments

Unlike in the case of several basic drugs, the incorporation of EtG is not affected by hair colour. This is due to the fact that EtG does not bind to melanin, as previously mentioned. Two studies have demonstrated this fact. Appenzeller et al. [115] collected hair samples from 21 subjects and separated the pigmented and white hair for each of the subjects. After analysing the samples for EtG, the results demonstrated that there was no significant intra-individual difference in the concentrations observed for the pigmented and white hair. This result was confirmed by an animal study using rats with areas of pigmented and non-pigmented hair [88].

Cosmetic treatments, on the other hand, can cause a considerable loss of EtG in hair. An *in vitro* experiment by Morini et al. [116] demonstrated that application of a commercially available bleaching solution completely removed EtG from hair. The results further showed that it was more likely that EtG was washed out from the cosmetically damaged hair rather than degraded chemically through reactions with oxidising agents, such as hydrogen peroxide, used in bleaching solutions. Similar results were obtained in another *in vitro* study, however, rather than EtG being totally removed from hair after bleaching, a mean decrease of 73.5% in EtG concentrations was observed [117]. The effects of using a temporary colouring dye and a perm treatment on hair were also investigated in this study. Whilst the colouring product did not appear to influence the EtG concentrations, the perm treatment caused a mean decrease of 95.7% in EtG concentrations, assumed to be from chemical degradation of EtG. These results are supported by *in vivo* studies that have demonstrated significantly lower EtG concentrations in hair that has been bleached [78, 118, 119]. These studies also show that the use of permanent colouring dye leads to a significant reduction in EtG concentrations.

Thermal hair straightening was demonstrated by Ettlinger et al. [120] to influence EtG concentrations in hair. Hair samples positive for EtG (n = 41) were treated *in vitro* with a hair straightener at 200°C for 1 min. Results indicated that, depending on hair colour, straightening either caused an increase or decrease of EtG. A mean increase of EtG was found in darker hair whilst a mean decrease of EtG was found in lighter hair after straightening. The authors suggest that this effect could be due to the dark hair samples being thicker and that EtG incorporated into thicker hair fibres is less denatured by heat. However, these are preliminary results and need to be confirmed by an *in vivo* study.

The influence of ethanol containing hair cosmetics on EtG levels in hair was investigated by Martins Ferreira et al. [104]. Results from this study indicated that hair cosmetics containing ethanol do not lead to the formation of EtG and therefore an increase in the

amount of EtG incorporated in hair. These findings were supported by another study in which the use of cosmetic products, such as hair spray, had no effect on EtG levels [78].

In summary, EtG levels are not influenced by hair colour or the use of ethanol containing hair cosmetics, but appear to be significantly reduced after bleaching, permanent dying or perming, leading to the potential reporting of false negative results. Therefore, as recommended in the consensus of the SoHT [75], it is important that the use and type of cosmetic hair treatment is documented during the collection of hair samples and considered during the interpretation of results.

Effect of other factors

Other factors known to influence ethanol metabolism, such as age, gender and body mass index (BMI), have also been investigated to determine the effect on hair concentrations of EtG. A study by Mornini et al. [121], however found that none of these factors (age, gender and BMI) significantly influenced the performance of EtG in hair. The role of gender differences on EtG was investigated in another study which also reported no influence of gender on the incorporation of EtG in hair [122]. On the contrary, significantly lower EtG levels in female hair samples, independent of cosmetic treatments, have also been reported [78, 118, 123]. Although, it is not made clear if these differences are as a result of a lower production of EtG in females or due to lower levels of alcohol consumption among females. In a recent study concentrations of EtG in hair were reported to be influenced by BMI [124]. Among individuals who consumed similar amounts of alcohol a significantly higher mean EtG concentration was observed in individuals with a BMI ≥ 25 (overweight and obese) compared to individuals with a BMI < 25 (underweight and normal-weight).

Non-head hair

Head hair is not always available and in these situations hair from other parts of the body (e.g. pubic, axillary, chest, arm, leg, beard hair) can be analysed. Although, slower growth rates as well as a larger percentage of hair in the telogen (resting) phase can make it difficult to estimate when alcohol was consumed [125]. Higher concentrations

of EtG have been observed in pubic hair compared to head hair [98, 101, 111]. The increased concentration of EtG observed in pubic hair is most likely due to incorporation from EtG positive urine [101]. For example, increased concentrations of EtG in pubic hair between 12 and 1370 pg/mg were reported for eight volunteers with head hair EtG concentrations below 10 pg/mg [111]. No EtG was found in the pubic hair of volunteers not exposed to alcohol. Therefore, whilst pubic hair cannot be used to distinguish between social and heavy drinking, it can be useful in assessing abstinence. Axillary hair contains lower amounts of EtG compared to head hair and is, therefore, also not suitable for assessing alcohol consumption [98, 125]. EtG concentrations in chest, arm and leg hair on the other hand are comparable to concentrations in head hair [98, 101, 125]. Chest hair appears to be the most similar to head hair and is the preferred alternative to head hair, although can usually only be collected from males.

1.4.2.4 Correlation between alcohol consumed and ethyl glucuronide in hair

The relationship between the amount of alcohol consumed and observed EtG concentrations in hair has been investigated in a number of studies, however, there are different opinions regarding whether a correlation between these two variables exists or not. In an early study by Skopp et al. [91] a lack of correlation between drinking behaviour and EtG levels in hair was found. For two out of the four patients identified to be current heavy drinkers, no EtG was detected in the hair. A similar result was reported in a study by Janda et al. [93] where 11 out of 42 known alcoholics had undetectable levels of EtG in hair samples. The false negatives reported in both of these studies could be attributed to the high limit of detection (LOD) of these two methods (2200 pg/mg [91] and 51 pg/mg [93]). Although, the removal of EtG during normal hair washing, due to the polar nature of EtG, and the impact of dyeing hair on EtG levels are provided as possible reasons for the false negative results reported in one of the studies [93].

More recent studies with increased sensitivity compared to earlier methods have also reported a poor correlation between levels of EtG in hair and alcohol consumption [80, 121]. A controlled alcohol-dosing study in humans was developed by Kronstrand et al.

[97] to investigate if total abstinence could be differentiated from a low or moderate consumption of alcohol according to EtG levels in hair. Over a period of three months, seven male volunteers consumed 32 g of ethanol daily, 14 female volunteers consumed 16 g of ethanol daily and 23 volunteers remained abstinent. Only four out of the seven males consuming 32 g of ethanol daily had measurable amounts of EtG in their hair (5 – 11 pg/mg), and for only one out of the 14 females drinking 16 g ethanol daily was EtG quantified (3 g/mg). Two of the abstinent volunteers had traces of EtG in their hair. Although the study has certain limitations the results are significant as they demonstrate the poor individual relationship between the amount of alcohol consumed and levels of EtG in hair. Furthermore, the consumption of 32 g of ethanol daily for three months resulted in EtG concentrations in hair that were much lower than 30 pg/mg and the consumption of 16 g of ethanol did not result in EtG concentrations higher than 7 pg/mg (the proposed cutoff for abstinence assessment).

On the contrary, another controlled study in humans demonstrated concentrations of EtG in hair to be consistent with the amount of alcohol consumed [126]. Healthy individuals (n = 30) were matched on age and gender and instructed to drink no alcohol (n = 10), 100 g alcohol per week (n = 10), or 150 g alcohol per week (n = 10) for three months. Median EtG concentrations in the non-drinking, 100 g per week and 150 g per week groups were 0.5 pg/mg, 5.6 pg/mg and 11.3 pg/mg respectively. Hair EtG concentrations for each of the three groups were significantly different from each other. EtG concentrations for eight of the participants consuming 100 g alcohol per week were less than 7 pg/mg and in light of these results the authors suggest that a cutoff lower than 7 pg/mg be considered to distinguish between abstinence and social drinking.

Appenzeller et al. [127] demonstrated a significant correlation between EtG concentrations determined from segmented hair samples and self-reported alcohol consumption. Blood samples analysed for traditional alcohol biomarkers, including GGT and MCV, showed no correlation to alcohol consumption or EtG levels in hair. Similar results have been reported in another study that compared EtG in hair with traditional alcohol biomarkers, including CDT and GGT [128]. Again, hair EtG concentrations were

significantly correlated with ethanol intake whereas traditional alcohol biomarkers were not. Further studies have been published that also report a correlation between the amount of alcohol consumed and EtG levels in hair [101, 113, 118, 122, 129], including one animal study [88].

The variability in EtG results presented in the literature may be related to a number of factors including differences in drinking behaviour, alcohol metabolism, physiology in hair growth, hair care as well as the use of cosmetic hair treatments [80]. Additionally, alcohol consumption measured by self-report may be inaccurate and biased.

1.4.3 Cocaethylene

When alcohol and cocaine (COC) are ingested at the same time an active metabolite of COC, cocaethylene (CE), is formed [130, 131]. COC is transformed into CE by hepatic carboxyltransferase which is also responsible for the formation of benzoylecgonine (BZE) [131]. In the presence of alcohol up to 17% of intravenous COC is transformed into CE, resulting in reduced BZE concentrations [132]. CE has been detected in hair [133–135], and also investigated as a biomarker in hair for excessive alcohol consumption [136–138]. However, its use as a biomarker for alcohol consumption is limited to instances of simultaneous cocaine use.

A comparison between the determination of EtG and CE in hair samples of COC users was performed by Politi et al. [139]. In total, 68 hair samples were analysed and using cutoffs of 4 pg/mg for EtG and 200 pg/mg for CE, 47 hair samples tested positive for EtG and 41 tested positive for CE. Out of the 47 hair samples positive for EtG, 12 tested negative for CE, and out of the 41 positive CE samples, six tested negative for EtG. No quantitative correlation was observed between concentrations of EtG and CE in the hair samples. The authors provide various explanations for the lack of correlation between the two alcohol markers including personal habits and patterns of ethanol and COC consumption, differences in the metabolism and mechanisms of incorporation of EtG and CE as well different effects on the markers by hair care and cosmetic treatments. A

positive CE result and a negative EtG result can occur in cases of moderate alcohol consumption by individuals with frequent cocaine use. Nonetheless, the detection of CE proves that both COC and alcohol have been used concurrently and can be used to support results from other alcohol markers [94].

1.5 Hair analysis as a measure of drug abuse

A majority of the research surrounding hair analysis is focused on the detection and quantification of drugs of abuse in hair. In a number of countries hair testing has gained value as an alternative or complimentary matrix for identifying drug use. As a result the applications for drug testing in hair are widespread and include workplace drug testing, drug-related deaths, drug-facilitated crime, prenatal drug exposure, re-issuing of driving licences and monitoring drug exposure [62]. Several comprehensive reviews have been published over the years on the topic of hair analysis for drugs of abuse and the range of analytical methods used [62, 140–144].

1.5.1 Techniques for the analysis of drugs of abuse in hair

The purpose of this section is not to give an in-depth review of the published analytical methods for the analysis of drugs of abuse in hair, but rather to provide a discussion of sample preparation procedures and LC methods that have been published regarding the drugs of abuse and their metabolites relevant to this study, namely; amphetamine (AP), methamphetamine (MP), COC, BZE, CE and methaqualone (MQL). No published methods for the determination of MQL in hair could be found in the literature.

Sample preparation

Wash procedures vary according to the type of solvent used, the duration of wash steps as well as the number and sequence of wash steps used. Acetone, dichloromethane and water are the most commonly used solvents for decontaminating hair samples. Wash procedures that have been used for all drug classes often include two washes with

organic solvents such as dichloromethane or a single short wash with methanol [140]. The SoHT recommends that laboratories investigate the effectiveness of their wash procedures in removing surface contamination from hair [56]. As a result, laboratories frequently keep the solvent from the last wash step to analyse for any remaining external contamination [145–148].

For the analysis of APs, decontamination steps with water and methanol [149], isopropanol and phosphate buffer [150], dichloromethane [147], 0.1% sodium dodecyl sulphate, water and methanol [151] have been described. For the analysis of COC different wash procedures have been evaluated to distinguish between external contamination and ingestion of COC [152–154]. Schaffer et al. [154] evaluated the effectiveness of an extensive wash procedure consisting of consecutive washes with isopropanol and phosphate buffer compared to a simple methanol wash procedure for removing COC contamination. The results demonstrated the effectiveness of the extensive wash procedure in removing all of the external contamination compared to the methanol procedure. The results of this study were supported by the results of another study using the same wash procedure [155]. Although, in the second study the need for specific reporting criteria to exclude false positive results was established. Contrary to these studies, it has also been demonstrated that even with using an extensive procedure consisting of wash steps with ethanol, phosphate buffer and water it was not possible to distinguish between external contamination and COC ingestion [153]. Dichloromethane has also been used to decontaminate hair samples prior to analysis for COC [133, 134, 156, 157].

An extraction procedure that is compatible with almost all drug classes is extraction with methanol. Hair samples, 10 – 50 mg, are generally incubated in 1 – 2 ml methanol in an ultrasonic bath. Incubation times usually vary between 5 to 18 hours. Ultrasonication causes disruption of the hair structure and methanol penetrates the hair matrix causing hair to swell and drugs to be released through diffusion. Methanol is hydrophilic and therefore dissolves neutral and hydrophilic substances. This approach of drug extraction, however, usually requires a clean-up step due to the high level of impurities

in the extract. Furthermore, drug recovery is often incomplete, resulting in lower recoveries compared to when other extraction procedures are used [62].

Digestion with sodium hydroxide (NaOH) is advantageous for the extraction of APs due to the basic environment, however COC is unstable under alkaline conditions and hydrolyses to BZE [140]. Acidic extraction procedures have the advantage of simultaneously extracting a range of substances for analysis including APs and COC and have subsequently been used in a number of multi-analyte methods [158–161]. However, partial hydrolysis of COC results from acidic conditions as well, which is a disadvantage when using this approach [140].

In the development of a screening method for 30 substances of abuse, including APs and COC and its main metabolites, three extraction procedures were evaluated [162]. The evaluated extraction procedures were: direct methanol extraction in an ultrasonic bath for 8 hours at 50°C, acidic extraction with hydrochloric acid (HCl) incubated for 18 hours at 50°C and alkaline digestion with NaOH incubated for 30 min at 100°C. For both the acidic extraction and alkaline digestion procedures an additional SPE clean-up step was performed. Interestingly, cleaner extracts and better recoveries were found with the direct methanol extraction.

Drugs of abuse can also be extracted from hair using mixtures of solvents [140]. A simple extraction method was developed by Kronstrand et al. [163] for the simultaneous extraction and analysis of several drugs of abuse in hair using LC-MS/MS. The extraction procedure simply involved direct addition of mobile phase A, made up of a 10:10:80 mixture of acetonitrile:methanol:20 mM formate buffer (pH 3), to hair samples followed by incubation for 18 hours at 37°C.

An alternative approach to extracting drugs from hair was developed by Miyaguchi et al. [151, 164] that involved a simultaneous pulverization and extraction step (micropulverized extraction). The initial method developed was for the analysis of MA using 2 mg hair samples [151]. Washed hair samples were pulverized for 5 min together

with extraction solvent followed by direct injection of the filtered extract into the LC-MS/MS system. This approach significantly reduced the extraction time from 18 hours to 5 min. Following this, a second micropulverized extraction method was developed for the determination of APs, COC and ketamine using 0.2 mg hair samples [164]. Favretto et al. [145] optimized the simultaneous pulverization and extraction method developed by Miyaguchi et al. [151, 164] and developed a method for the extraction of 28 different drugs from hair. A 5 min simultaneous pulverization and extraction step that did not require any further clean-up steps was also used in this method. The method was validated using 2.5 mg hair samples and reached LLOQ values of between 100 and 500 pg/mg depending on the compound. Additional methods using micropulverized extraction techniques have since been developed for the determination of a range of drugs in hair [146, 148].

Liquid Chromatography

Drug users often tend to use more than one type of drug at a time and therefore multi-analyte methods capable of detecting and quantifying a range of drugs and their metabolites are useful. Most hair analysis methods developed nowadays make use of LC-MS/MS techniques, and a number of multi-analyte LC-MS/MS methods have been published [141]. An advancement of LC systems is ultra high performance LC (UHPLC) or ultra performance LC (UPLC). These systems use analytical columns with very small particle sizes that are able to tolerate high pressures. Chromatographic performance is increased as a result and using this technique multiple analytes can be separated in short run times [141].

Montesano et al. [148] achieved the separation of 96 drugs from different drug classes within a total run time of 19.5 min. All of the compounds eluted between 1.40 and 16.05 min. Analytes were extracted from 10 mg decontaminated hair samples by a simultaneous pulverization and extraction step followed by overnight incubation with extraction medium. The extracts were filtered, diluted with deionized water and injected directly into a UPLC-MS/MS system. Compared to conventional HPLC, UPLC systems generate narrow peaks providing enhanced chromatographic resolution. As a

result, peak detection can be challenging, especially in methods with multiple analytes. The MS acquisition method was optimized in this study in order to ensure that a sufficient number of data points were collected to obtain accurate descriptions of chromatographic peaks. The LODs for the analytes were in the low pg/mg range and sensitive enough to allow for the detection of a single drug exposure.

High-resolution mass spectrometry (HRMS) techniques such as time-of-flight (TOF) and Orbitrap MS offer the advantage of accurate mass measurement. These techniques coupled to either LC or UHPLC have been successfully used for the determination of a range of drugs of abuse in hair [141]. An UPLC-TOF-MS method has been developed and validated for the simultaneous screening and quantification of 52 common drugs of abuse and pharmaceuticals in hair [165]. The total run time was 17 min. LODs ranged between 10 and 100 pg/mg. This study demonstrated the suitability of the TOF instrument to be used for both screening and quantification purposes.

More recently, a LC-HRMS method for the simultaneously screening and quantification of over 170 drugs of abuse and pharmaceuticals in hair was developed [166]. The simple extraction procedure involved overnight incubation of decontaminated hair samples (30 mg) in an ultra-sonic bath with methanol. The extract was injected directly into the LC-HRMS system, and the total run time was 14.5 min. Despite the simple sample preparation procedure, matrix effects were negligible and suitable sensitivity was achieved with LODs varying from 2 to 30 pg/mg. Even though the method was intended for screening purposes a further fragmentation experiment allowed for the confirmation of suspected positive cases. An advantage of the Orbitrap mass spectrometer is that when operated in full-scan mode the accurate mass of all ionizable compounds eluted from the HPLC run are stored. As a result future retrospective screening can be performed to search the stored data for new drugs or metabolites [141].

1.5.2 Data Interpretation

Along with accurately interpreting hair analysis results for EtG, the interpretation of results for drugs of abuse in hair can also become challenging and should be done with caution. An important consideration is that drug concentrations in hair do not necessarily reflect the amount of drug ingested or the frequency of drug use. Instead, research suggests that there is limited correlation between dose and drug concentrations in hair [62, 141, 167]. The most likely explanation for this being inter-individual variations in drug metabolism and incorporation. Several other factors, some of which are discussed below, also influence drug levels in hair and as such detection of a drug is not always sufficient to identify drug use [141].

External Contamination

Unlike blood or urine, hair is exposed to the external environment and is therefore open to environmental contamination. This is particularly relevant in the case of drugs that can be smoked such as COC, AP, MP, marijuana and heroin. Passive exposure to drugs can result in false positive results. The possibility of reporting false positive results is one of the main limitations of hair analysis [62, 141]. There are generally three complementary approaches that laboratories use to determine whether a positive test result is due to active drug use or as a result of passive exposure. The first is that hair samples are decontaminated by washing hair prior to analysis. The second approach has to do with the detection of relevant drug metabolites in the hair sample, and thirdly, cutoff values are used [168].

The use of wash criteria has been suggested in addition to decontamination procedures to distinguish between drug use and external contamination [155, 168–170]. These criteria involve calculating ratios between drug concentrations detected in the solution from the last wash and in the hair sample. Greater drug concentrations in the last wash compared to in the hair generally indicate external contamination, whereas when drugs are detected in hair and not in the last wash, drug use is assumed. Whilst the successful use of wash criteria to exclude false positive results have been reported [155, 168, 170], they are not always adequate in identifying cases of external contamination [171].

The detection of drug metabolites, specifically those that form from endogenous metabolism, is another approach that is used to avoid false positive results. The SoHT has established cutoff values for commonly abused drugs and their metabolites [56], and also recommends the use of metabolite-to-parent drug ratios [172]. For example, a positive sample for COC should have a BZE/COC ratio greater than 0.05. However, it is important to note that metabolites can sometimes be formed by non-metabolic processes. The hydrolysis of COC to BZE is an example [169]. Stout et al.[173] studied *in vitro* COC contamination in hair by contaminating hair samples collected from five individuals with COC HCl. The concentration of COC analytes were determined over a ten week period during which the hair was subjected to regular shampooing. Results indicated that the BZE/COC ratios increased significantly over the ten week study. Moreover, from 21 days after the hair samples were contaminated until the end of the study, the mean BZE/COC ratio for all of the hair samples was greater than 0.05. Therefore, use of the BZE/COC ratio was ineffective in this study in avoiding false positive results.

Effect of hair colour

As previously discussed, the incorporation of basic drugs in hair is influenced by the melanin content of hair. Hair colour is determined by the amount of melanin and darker hair contains more melanin compared to hair that is lighter in colour. Many drugs of abuse, including APs and COC, are basic and as a result, higher concentrations of these drugs and their metabolites are observed in darker hair [141]. This effect was demonstrated in controlled dose study using rats [65]. Male Long-Evans rats, which contain both black pigmented and white non-pigmented hair, were injected with either AP or *N*-acetlyamphetamine, a non-basic analogue of AP, once a day for five days. The rats were shaved prior to the study and again 14 and 28 days after the initial drug administration. The shaved hair samples were separated into white and black samples and analysed for AP and *N*-acetlyamphetamine. The concentration of AP in black hair collected from rats dosed with AP was 6.44 ng/mg (n = 8). This was significantly different from the concentration of AP in white hair collected from the same rats (2.04 ng/mg).

However, no significant difference was observed between *N*-acetlyamphetamine concentrations in black (0.87 ng/mg) and white (0.83 ng/mg) hair collected from rats dosed with *N*-acetlyamphetamine (n = 8). The relationship between the incorporation of AP and MP in human hair and melanin content has also been investigated [174, 175]. In these studies melanin content was measured using spectrophotometry and concentrations of AP and MP in hair were measured using GC-MS. Results from the analyses showed an increase in the levels of AP and MP detected with increased melanin content.

Studies investigating the influence of melanin content on the incorporation of COC in both animal [176] and human [177] hair have also demonstrated increased concentrations of COC in darker hair compared to lighter hair. The higher accumulation of basic drugs in darker hair introduces the potential for not only colour bias, but racial bias as well since different races tend to have a common or specific hair colour [141]. In a controlled dose study isotopically labelled COC (COC-d_5) was administered either intravenously or intranasally to 25 human volunteers, four of which were non-Caucasian [178]. Hair samples were analysed for COC-d_5 by GC-MS and results indicated that non-Caucasians incorporated two to 12 times more COC-d_5 than Caucasians. This bias towards increased drug concentrations in non-Caucasian hair has been further demonstrated in other studies on COC [177, 179, 180] as well as codeine [66] concentrations in hair.

Effect of cosmetic treatments

The chemicals used in cosmetic hair treatments such as bleaching, dyeing and perming, change the chemical composition of hair which affects the stability of drugs incorporated in hair [181]. Jurado et al. [182] studied the effects of bleaching and dyeing on the concentrations of a range of drugs of abuse, including COC, detected in hair. Hair samples were collected from drug users who either bleached or dyed their hair. Treated and untreated portions of hair collected from each subject were separated and analysed for drugs of abuse using GC-MS. The results showed that for each of the subjects the drug concentration in the treated portion of hair was less than in the untreated hair.

Concentrations of COC measured in treated hair were 30.6% to 76.0% less than the concentrations measured in untreated hair. Moreover, the decrease in the measured concentrations was higher in hair that had been previously bleached compared to dyed hair, suggesting that hair that has been more damaged will have lower drug concentrations. Similarly, bleaching treatments also result in decreased concentrations of AP and MP in hair [183].

On the other hand, damage caused by cosmetic hair treatments increases the porosity of hair causing it to become more accessible to external contamination [181, 184]. This effect was demonstrated by a recent *in vitro* study investigating COC incorporation in hair damaged by cosmetic treatments [156]. Drug free hair (n = 7) was subjected to either bleaching, dyeing or straightening and subsequently soaked in a COC solution for 60 min, along with untreated hair, to mimic external contamination. After soaking hair samples were washed and analysed for COC by GC-MS. The untreated and straightened hair did not show any significant uptake of COC after the 60 min soaking, whereas considerable COC uptake was observed for the bleached and dyed hair. Measured COC concentrations in these treated hair samples were above the 500 pg/mg cutoff value.

The results from the studies discussed above highlight the importance of taking cosmetic treatments, and the various ways in which they can possibly influence drug levels, into consideration when interpreting hair analysis results.

1.6 Hair analysis for antiretrovirals as a measure of adherence

In addition to the widespread applications of hair analysis in the field of forensic toxicology hair analysis has, more recently, gained increased recognition in the field of clinical toxicology. More specifically, the potential of hair analysis for TDM is becoming well-established. An advantage of monitoring drug levels in hair compared to plasma is that hair levels reflect drug exposure over weeks to months and therefore have the potential of assessing long-term adherence. Moreover, drug levels in hair are not subject

to bias from the 'white-coat effect' or inaccurate self-report about when the last dose was taken [27]. Measuring drug levels in both plasma and hair samples collected from patients can provide further insight into the adherence patterns of patients [185]. Drug levels in plasma will reflect recent drug exposure whereas drug levels in hair will reflect average drug exposure over a longer period of time.

Measuring drug levels in hair is also useful in predicting treatment outcomes and several studies have reported ARV concentrations measured in hair to be strong predictors of virologic response to treatment. This relationship has been demonstrated for the ARVs atazanavir [186, 187], indinavir [188, 189], lopinavir [185, 187], EFV [190, 191], nevirapine [192] and lamivudine [193]. More recently, hair has been investigated as a potential adherence measure for pre-exposure prophylaxis (PrEP) involving the determination of tenofovir and emtricitabine levels in hair [194–196].

1.6.1 Methods for the analysis of antiretrovirals in hair

Sensitive LC-MS/MS methods have been published for the determination of the ARVs EFV, lopinavir, and ritonavir [197], nevirapine [198], abacavir and tenofovir [199] in hair. Most PIs and NNRTIs are lipophilic drugs with basic properties and are therefore well incorporated in hair and present at high levels [200]. As a result, only very small quantities of hair are needed in order for these drugs to be detected (∼2 mg). Nevirapine was easily detected after extraction from a single strand of hair 1.5 cm in length (0.1 mg) [198].

In general, similar sample preparation procedures have been followed for the analysis of the ARVs listed above in hair. In most cases hair samples are not decontaminated prior to extraction. Since ARVs are taken orally and are not volatile drugs the possibility of external contamination is thought to be low [198]. The effect of pulverizing hair samples on the amount of drug extracted was investigated for the ARVs EFV, lopinavir, ritonavir [197] and nevirapine [198]. The results showed similar levels of drug in both powdered and cut hair and as a result pulverizing hair samples was found to be

unnecessary. Moreover, very small samples of hair were used (~ 2 mg) and cutting hair samples into 1 mm segments was easier and less time consuming compared to pulverizing hair samples. However, the sample preparation procedure for the analysis of abacavir and tenofovir in hair involves a decontamination step with dichloromethane followed by pulverization of hair samples (50 mg) prior to extraction [199]. Generally, ARVs are extracted from hair during an overnight incubation in a water bath with methanol and following a LLE clean-up step extracts are injected into a LC-MS/MS system for analysis [197–199].

Currently, the only published method for the quantification of EFV in hair is a method that was developed by Huang et al. [197] in 2008. EFV as well as lopinavir and ritonavir were extracted from 2 mg hair samples using the methanol extraction procedure described above. The method was validated for EFV from 0.05 – 20 ng/mg hair. The validated method was highly specific and sensitive with negligible matrix effects. In addition to quality controls (QCs) prepared from spiking ARV free hair with the analytes of interest, authentic QCs prepared from hair samples collected from HIV-patients on ART were also used to demonstrate the reproducibility of the assay. Authentic QCs provided a better representation of patient samples since spiking hair with drugs does not mimic the processes by which drugs are incorporated in hair. The validated method was applied to analyse 68 hair samples collected from patients on EFV-based HAART. The median EFV level in the hair samples collected from patients who responded to HAART (n = 54) was 3.4 ng/mg, whereas the median EFV level in the hair of non-responders (n = 14) was significantly lower (0.68 ng/mg).

1.6.2 Antiretrovirals in hair and virologic outcomes

Bernard et al. [188] first showed in 1998 that the mean concentration of indinavir measured from hair collected from HIV-infected patients responding to HAART (n = 19) was significantly higher than in patients who did not respond to HAART (n = 11) (17.85 ng/mg and 8.01 ng/mg respectively). These results were supported by a follow up study performed by the same group using a larger patient sample [189]. Once again the results

indicated that the mean concentration of indinavir measured in the hair of responders (n = 65) was significantly higher than in the non-responders (n = 24) (24.4 ng/mg and 12.9 ng/mg respectively). Another study with 43 HIV-infected patients showed concentrations of indinavir in hair to be more strongly correlated with virologic suppression compared to plasma indinavir levels [201].

Gandhi et al. [187] was the first to quantify the PIs lopinavir, ritonavir and atazanavir in small samples of hair (between 1 – 5 mg) using similar LC-MS/MS methods used to measure plasma levels. The relationship between PI levels in hair samples collected from 224 patients and virologic success was analysed. Patients were either starting a lopinavir/ritonavir (n = 70) or an atazanavir-based (n = 154) regimen. The results demonstrated that PI levels in hair were the strongest independent predictor of virologic success. Hair levels were categorised into tertiles and adjusted odds ratios were calculated controlling for self-reported adherence, age, race, starting viral load and CD4 count, total time on drug and prior ARV experience. The adjusted odds ratio for virologic success was 39.8 (95% CI 2.8 – 564, P = 0.006) for patients with lopinavir hair concentrations in the top tertile (> 1.9 ng/mg) compared to the bottom tertile (≤ 0.41 ng/mg). For patients with atazanavir hair concentrations in the top tertile (> 3.4 ng/mg) the adjusted odds ratio for virologic success was 7.74 (95% CI 2.01 – 29.7, P = 0.003) compared to those in the bottom tertile (≤ 1.2 ng/mg).

A cross sectional study of virologic failure in adult patients attending treatment clinics in Cape Town, South Africa was conducted by Van Zyl et al. [185]. Patients treated with a second-line regimen of lopinavir boosted with ritonavir (n = 93) were recruited for the study and of these 50 were included in a nested case control study (25 cases with virologic failure and 25 controls). The case control study was used to assess the value of hair and plasma lopinavir concentrations. Plasma samples were analysed for lopinavir using a validated LC-MS/MS method at the University of Cape Town. However, at the time of the study, hair analysis was only available at a laboratory in San Francisco, California and the hair samples had to be sent away for analysis. Hair samples were analysed for lopinavir and ritonavir using a modified LC-MS/MS method [187, 197].

Lopinavir plasma and hair concentrations were compared in patients with or without virological failure (Figure 1.5) and were reported to be significantly lower in patients with virological failure. In concluding the authors found the use of both plasma and hair lopinavir concentrations useful in determining the cause of virologic failure, which in this instance was due to poor drug exposure most likely as a result of poor adherence [185].

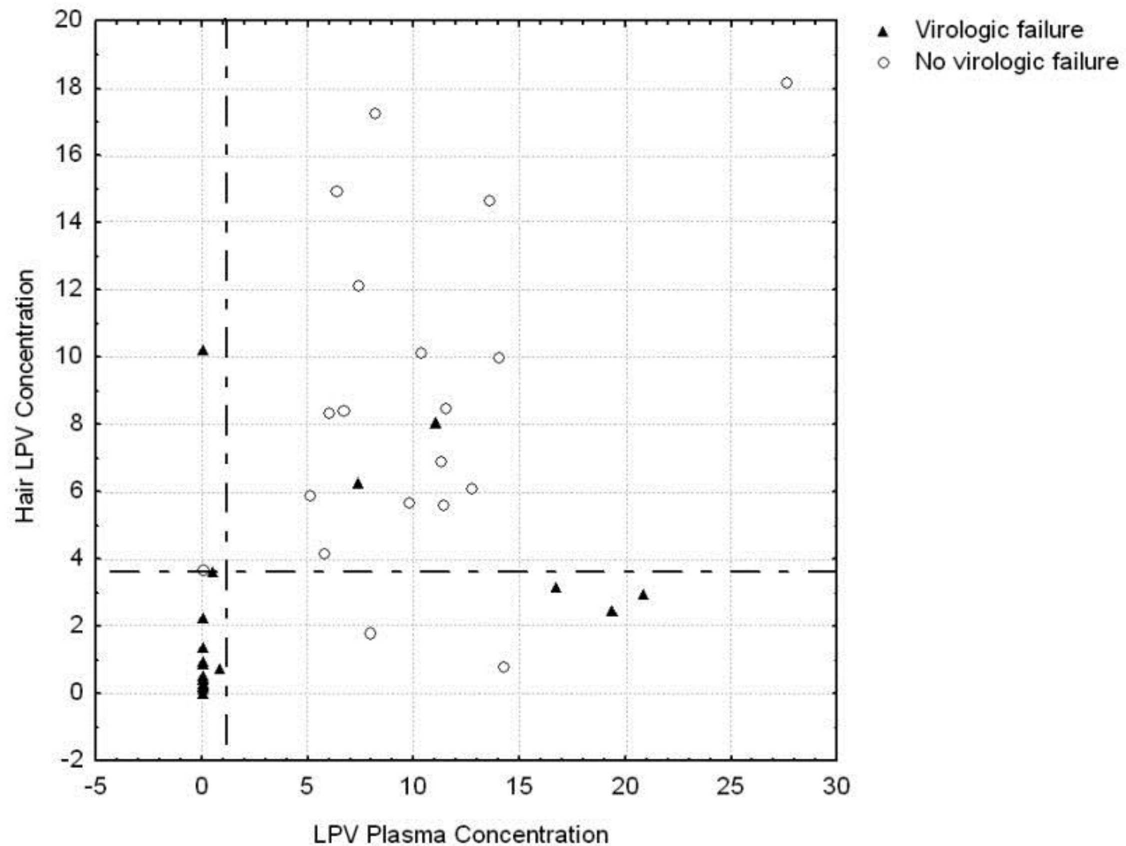

Figure 1.5 Scatterplot of lopinavir (LPV) hair (ng/mg) and plasma concentrations (µg/ml) in patients with (triangles) and without virologic failure (open circles). Dashed lines indicate the respective concentration cutoffs determined by receiver operating characteristic analysis: lopinavir plasma concentration of 1 µg/ml and lopinavir hair concentration of 3.63 ng/mg. Copyright © 2011, Wolters Kluwer Health, Inc., van Zyl et al. [185].

1.6.3 Efavirenz related studies

In a study with 121 HIV-infected women, Gandhi et al. [190] assessed the correlation between EFV levels in hair and three other common measures of drug exposure: self-reported adherence, a single EFV plasma concentration and intensive pharmacokinetic measurements. Additionally, the relationship between each of these measures and virologic outcomes was assessed. The results indicated that EFV concentrations in hair were weakly correlated with self-reported adherence and single EFV plasma concentrations, but strongly correlated with intensive pharmacokinetic measurements. Moreover, only the hair EFV levels were statistically correlated with an undetectable viral load.

In another study, EFV concentrations were measured in hair samples collected from HIV-infected pregnant and breastfeeding women in Uganda [191]. Hair samples were collected at 30 – 34 weeks gestation and at 10 – 25 weeks postpartum. EFV concentrations were measured using a previously described LC-MS/MS method [197]. Viral loads were measured at delivery and 24 weeks postpartum. In multivariate models the strongest predictor of viral suppression at delivery and 24 weeks postpartum was EFV hair concentrations.

Röhrich et al. [202] demonstrated the feasibility of using hair samples for testing EFV concentrations to determine long-term drug exposure in South Africa. Additionally, variations in CYP2B6 were characterized in order to investigate the influence of polymorphisms in this gene on long-term EFV exposure. Female HIV-infected patients receiving EFV-based treatment at a HIV clinic in the Western Cape, South Africa were recruited for the study. The participants were either from the South African Black (SAB) (n = 77) or Cape Mixed Ancestry (CMA), also known as Coloured, (n = 43) populations. Hair samples collected from the patients were sent to a laboratory in San Francisco, California where they were analysed for EFV using a validated LC-MS/MS method [197]. The median EFV hair concentration for the combined SAB and CMA populations was 5.60 ng/mg (IQR: 3.93 – 7.06 ng/mg). Unlike in previous studies [190, 191], EFV hair levels were not associated with virologic outcomes. Although, a significant limitation of

the study was the variable timing of the collection of hair samples relative to viral load measurements. Viral load measurements were only taken at six month intervals and it is possible that EFV levels in hair did not correlate with exposure during the period of time in which the viral loads were measured thereby limiting the ability of the study to assess the association between hair levels and virologic outcomes. Regarding the influence of CYP2B6 genetic polymorphisms on EFV levels in hair, results from the study demonstrated that metaboliser phenotype was a statistically significant predictor of EFV levels in hair. Extensive and intermediate metabolisers demonstrated, on average, an estimated 57% (95% CI 35% - 71%) and 55% (95% CI 33% - 69%) lower EFV concentrations in hair, respectively, compared to slow metabolisers [202]. The results from this study are significant as they emphasise the effect that genetic variations in CYP2B6 has on long-term EFV exposure within the SAB and CMA populations. Furthermore, this was the first study to use hair samples to monitor EFV levels in HIV-infected patients in South Africa.

1.7 The importance of establishing methods for hair analysis in South Africa

The HIV pandemic and substance abuse are two major health challenges facing South Africa at the moment. Moreover, it is evident from the literature that substance abuse is prevalent among people who are infected with HIV and can be associated with non-adherence to ART, increased HIV disease progression and poor treatment outcomes. In order for interventions focused on reducing substance abuse among patients attending HIV clinics to be effective the extent of substance abuse among these populations needs to be well documented. However, measuring substance abuse, specifically alcohol use, can be challenging. Previously, self-report measures have frequently been used to measure alcohol and/or drug use. Self-report measures tend to be inaccurate as they are subject to recall and social desirability bias. Additionally, different self-report measures have previously been used, which makes it difficult to compare results

between studies. Therefore, objective measures of substance abuse are needed in order to accurately measure the extent of substance abuse among HIV-infected patients.

The analysis of hair as a biological matrix for drugs within the fields of both forensic and clinical toxicology has been well-established in many countries, which is evident from the vast amount of literature that has been published on this topic. However, up until recently, methods for hair analysis were not available in South Africa and the potential of this matrix as an objective measure of substance abuse has not yet been explored. In the clinical context, hair analysis offers the advantage of measuring long-term adherence to medication. As discussed in the literature, accurately measuring adherence can be complicated and despite the many different methods available there is no gold standard. Recently, hair has been used as a biological matrix for measuring ARV levels in order to monitor adherence and few studies have demonstrated the feasibility of analysing hair samples collected from South African patients for ARVs. However, at the time of the studies, hair analysis was not yet available in the country and hair samples collected for these studies had to be sent away to laboratories in America for analysis.

The hair analysis methods developed and validated in this study will allow for long-term monitoring of exposure to ARVs as well as provide objective measures needed to accurately assess the effect of substance abuse on ART adherence. Furthermore, this study will aid in establishing this methodology within South Africa so that in the future hair analysis for drugs can be used to advance the field of toxicology in the country.

1.8 Study aim and objectives

1.8.1 Aim

To evaluate whether the analysis of hair for the presence of drugs and/or metabolites can be used as an objective measure of substance abuse and ARV adherence among HIV-infected patients in South Africa, in order to assess the relationship between substance abuse and adherence.

1.8.2 Objectives

- Develop and validate a quantitative LC-MS/MS method for the determination of EtG in hair
- Develop and validate a qualitative LC-MS/MS method to screen for drugs of abuse in hair
    - Apply the methods to patient hair samples to determine the prevalence of alcohol and drug use within a population of HIV-infected patients
- Develop and validate a quantitative LC-MS/MS method to determine EFV levels in hair
    - Apply the method to patient hair samples to evaluate the usefulness of using hair analysis in monitoring adherence
- Assess the relationship between substance abuse and adherence within the study population by analysing data obtained from the analysis of the hair samples in the context of additional data obtained from the broader adherence study

2 Quantitative determination of ethyl glucuronide in hair

2.1 Introduction

EtG is a direct metabolite of ethanol formed from the conjugation of ethanol with glucuronic acid [82], and can only be formed following alcohol consumption. This highly polar and hydrophilic metabolite represents less than 0.06% of the amount of ethanol ingested [83]. EtG can be detected in blood and urine over a longer period of time than ethanol; it is detectable in blood for up to 8 hours and in urine for up to 80 hours [86, 87]. EtG also accumulates in hair, however due to its acidic properties (pKa = 3.21) only very small amounts are detected in hair [80].

Since the first results from the extraction of EtG from hair were published in 2000 [91, 92], EtG has been established as a valuable biomarker in hair that can be used to detect and quantify alcohol consumption over a long period of time. Cutoff values have been suggested by the SoHT from which abstinence and chronic excessive alcohol consumption (also referred to as heavy drinking or alcohol abuse) can be assessed according to the concentration of EtG detected in the hair. Chronic excessive alcohol consumers, or heavy drinkers, generally consume an average of 60 g or more of pure ethanol per day over several months [39, 75].

In the 2014 consensus of the SoHT, a cutoff of ≥ 30 pg/mg EtG in hair was recommended to identify chronic excessive alcohol consumption, and a cutoff < 7 pg/mg was recommended for abstinence assessment [75]. These cutoff values remained unchanged in the most recent revision of the SoHT consensus on alcohol markers in hair in 2016 [76, 203]. Despite the ≥ 30 pg/mg cutoff recommended by the SoHT, there is no general consensus and as such various cutoff values have been suggested to identify heavy drinking. Cutoff values of 23 [127], 25 [80], 27 [121], 30 [78] and 50 [111] pg/mg have been proposed and applied by various authors.

In addition, the results from a recent study by Salomone et al. [204] suggest that when hair is pulverized prior to extraction, as is recommended by the SoHT [75], an increased cutoff value of 40 pg/mg for heavy drinking might be more reliable. Results from the

study indicate that increased concentrations of EtG are observed when hair is pulverized rather than cut into small pieces prior to extraction. By pulverizing hair into a fine powder the structure of the hair is destroyed thereby increasing the surface area in contact with the extraction solvent. Several studies have reported similar results when comparing the effect that pulverizing hair samples has on the amount of EtG extracted [105–107].

Several analytical methods have been developed and validated for the analysis of EtG in hair. Most published methods are based on LC-MS/MS techniques as sample preparation is easier and no derivatization step is required [74]. The first fully validated method for the analysis of EtG in hair by LC-MS/MS was published in 2006 by Morini et al. [99]. A simple extraction method with no clean-up step and direct injection of the extraction solvent was used. A LOD and LLOQ of 2 and 3 pg/mg respectively were obtained using 100 mg of hair. More recent methods have also reported a LLOQ of 3 pg/mg, using only 30 mg of hair [100, 114], whereas Kronstrand et al. [97] validated a method with a LLOQ of 2 pg/mg using 30 mg of hair. Despite the numerous methods and validation data described in literature, there is no consensus on the preferred wash and extraction methods to be used when preparing hair samples for EtG analysis.

Additionally, there are certain limitations associated with the analysis of hair for EtG and therefore, in most cases, results should be interpreted with caution taking into consideration all of the relevant factors surrounding a particular case. For example, the use of thermal hair straightening tools might influence the concentration of EtG [205]. Cosmetic treatments such as bleaching, colouring and perming can result in significant amounts of EtG to be lost from the hair [78, 116–118].

Moreover, studies have suggested that normal hair hygiene could result in EtG being lost from hair [121, 206]. In one study aimed at investigating the stability of EtG in hair, overall lower levels of EtG were determined in older hair segments when compared to the more recent segments of hair. The authors of the study attributed this observed trend in the EtG levels to EtG being removed from the hair during normal hair hygiene

practices [206]. Conflicting views are reported in another study that showed no decrease in EtG levels when comparing the percentage of EtG positive samples for longer hair segments compared to shorter hair segments. The authors of this study, therefore, suggest that the washout effect plays a very minor role, if any, in the determination of EtG in hair [207].

The main aim of this chapter is to describe the development and validation of a method for the extraction and quantitative determination of EtG in hair using LC-MS/MS. The method was successfully applied to authentic samples of hair in order to identify participants in a study who were heavy consumers of alcohol. The validated method presented in this chapter is the first such method to be developed in South Africa. The results from the sample analysis provide objective insight into the drinking behaviour of HIV-infected patients in South Africa.

2.2 Materials and Methods

2.2.1 Collection and storage of hair samples

2.2.1.1 Blank and EtG positive samples

EtG free hair, collected from a child, was used during method development, preparation of calibration standards and QCs and validation experiments. Seven additional sources of EtG free hair for matrix effects and specificity experiments were collected from adult teetotallers. EtG positive hair was collected from volunteers with a known history of alcohol consumption, and in most cases alcohol abuse. The authentic, EtG positive hair was used during method development experiments. Hair collected from one of the volunteers was used throughout the study as an authentic QC.

A hair repository was registered with the University of Cape Town, Faculty of Health Sciences, Human Research Ethics Committee for the collection and storage of hair samples (reference R026/2015). Volunteers provided verbal consent to their hair being

collected. All hair samples registered in the repository were stored in paper envelopes at room temperature.

2.2.1.2 Study samples

Hair samples to be analysed for the purposes of the broader adherence study were collected from 135 HIV-infected patients (9 males and 126 females) who commenced ART at a treatment centre in Gugulethu, Cape Town. A total of 257 hair samples were collected over three visits to the centre at weeks 16 (n = 93), 32 (n = 75) and 48 (n = 89). Hair samples were cut from the posterior vertex region of the head as close to the scalp as possible and placed into paper envelopes. Proximal and distal segments of the hair were clearly marked. Hair samples were stored at room temperature until analysis.

Informed consent was obtained from all study participants. The collection and analysis of hair samples for the study was approved by the University of Cape Town Faculty of Health Sciences Human Research Ethics Committee (reference 101/2015 and 387/2015).

2.2.2 Chemicals and reagents

EtG (1 mg/ml in methanol) and its deuterated analogue (EtG-d5) (100 µg/ml in methanol), used as internal standard, were purchased from Cerilliant (Texas, USA). Working solutions were prepared by diluting EtG to 10 µg/ml, 100 and 10 ng/ml, and the internal standard to 1 µg/ml, 100 and 10 ng/ml with methanol. All reference and working solutions were stored below -20°C. Methanol and dichloromethane (gradient grade) were purchased from Merck (Darmstadt, Germany) and acetonitrile (LC-MS grade) was purchased from Honeywell International Inc. (Mexico City, Mexico). Formic acid (~ 98%) was purchased from Fluka Analytical (Buchs, Switzerland). Deionized water was prepared using a Synergy Water Purification System purchased from Merck Millipore (Massachusetts, USA).

2.2.3 Sample preparation

2.2.3.1 Wash method

Hair samples (20 mg) were washed by adding 1 ml of dichloromethane to each of the samples. The samples were vortex mixed briefly for 10 s and ultrasonicated for 5 min after which the dichloromethane was removed. The samples were allowed to air dry at room temperature.

A second wash method, including a methanol step, was investigated during the method development phase using authentic, EtG positive, hair. After the removal of the dichloromethane in the method described above, 1 ml of methanol was added to the hair samples. The samples were vortex mixed briefly for 10 s and the methanol removed. After the methanol wash step, the samples were allowed to air dry at room temperature.

For analysis of the wash solvents, the organic solvent was collected, 300 µl was removed, evaporated to dryness and reconstituted in 300 µl water with internal standard (10 ng/ml). The reconstituted samples (10 µl) were injected into the LC-MS/MS system for analysis.

2.2.3.2 Extraction method

For extraction of EtG, 20 mg samples of washed hair were pulverized into a fine powder using the Omni Bead Ruptor 24 (Omni International Inc., Georgia, USA). Four metal balls (2.4 mm) were added to weighed samples of hair in 2 ml screw-cap tubes. The metal balls and screw-cap tubes were purchased from Omni International Inc. (Georgia, USA). Samples were pulverized at a speed of 6 $m.s^{-1}$ for four cycles of 60 s each with a dwell time of 30 s after each cycle. A representative sample of hair before and after being pulverized is shown in Figure 2.1.

Figure 2.1 Authentic sample of hair, 20 mg, before (left) and after (right) pulverization using four metal balls at 6 $m.s^{-1}$ for four cycles of 60 s each.

After pulverizing the metal balls were removed and 300 µl water with internal standard (10 ng/ml) was added. Samples were vortex mixed briefly for 10 s, ultrasonicated for 15 min and the extract filtered through a 0.45 µm filter under vacuum (Merck Millipore, Massachusetts, USA). The clear extract (10 µl) was injected into the LC-MS/MS system for analysis.

During the method development phase various extraction conditions were investigated before deciding on the extraction method described above. The amount of EtG extracted after pulverizing samples of hair compared to cutting hair into smaller pieces was investigated using four samples of authentic, EtG positive, hair that was washed according to the method previously described. Aliquots of hair (20 mg) (n = 3) from each of the four EtG positive samples were cut into smaller pieces and extracted using water as described above. The amount of EtG extracted was compared to the amount of EtG extracted from aliquots of hair (20 mg) (n = 3) from each of the four EtG positive samples that were pulverized and extracted according to the methods described above.

Additional experiments were performed to investigate the effect of an overnight incubation step and a 15 min compared to a 2 hour ultrasonication step on the amount of EtG extracted. Authentic, EtG positive hair, from a volunteer, was washed and pulverized according to the methods described above. In one experiment samples of the authentic hair were incubated in water overnight at room temperature. After incubation the hair was ultrasonicated for either 15 min or 2 hours, after which the extracts were

filtered before analysis, as described above. This experiment was repeated on hair that had not been incubated in water overnight and only subjected to a 15 min or 2 hour ultrasonication step after adding the water. Each experiment was performed in triplicate and the extracts analysed to compare the amount of EtG extracted in each of the experiments.

2.2.4 LC-MS/MS Conditions

EtG was analysed using an Agilent Technologies 1260 Infinity Liquid Chromatography system (California, USA) coupled to an AB SCIEX Q Trap 5500 mass spectrometer (SCIEX, Massachusetts, USA). Chromatographic separation was achieved using an Agilent Poroshell 120 C18 column (2.7 µm, 4.6 x 50 mm) (Agilent Technologies, California, USA) kept at 50°C. Water with 0.1% formic acid was used as mobile phase A and acetonitrile with 0.1% formic acid as mobile phase B. The system was run using a gradient program starting at 2% phase B and increased to 4% in 1 min. From 1 to 3 min phase B was increased to 70% where it was kept for a minute. At 4.1 min phase B was decreased back to 2% where it was kept for 3 min. The total run time was 7 min at a constant flow of 500 µl/min.

The mass spectrometer was set to electrospray ionization in negative ion mode and analyte detection was performed in the multiple reaction monitoring mode (MRM). The mass spectra for EtG (Figure 2.2) and EtG-d5 (Figure 2.3) were obtained by direct infusion of each of the reference standards into the mass spectrometer. Reference standards were diluted to 100 ng/ml in 50% phase A and 50% phase B. EtG was monitored using two transitions, 221 → 75.0 (quantifier ion) and 221 → 85.0 (qualifier ion), and EtG-d5 was monitored at 226 → 74.9.

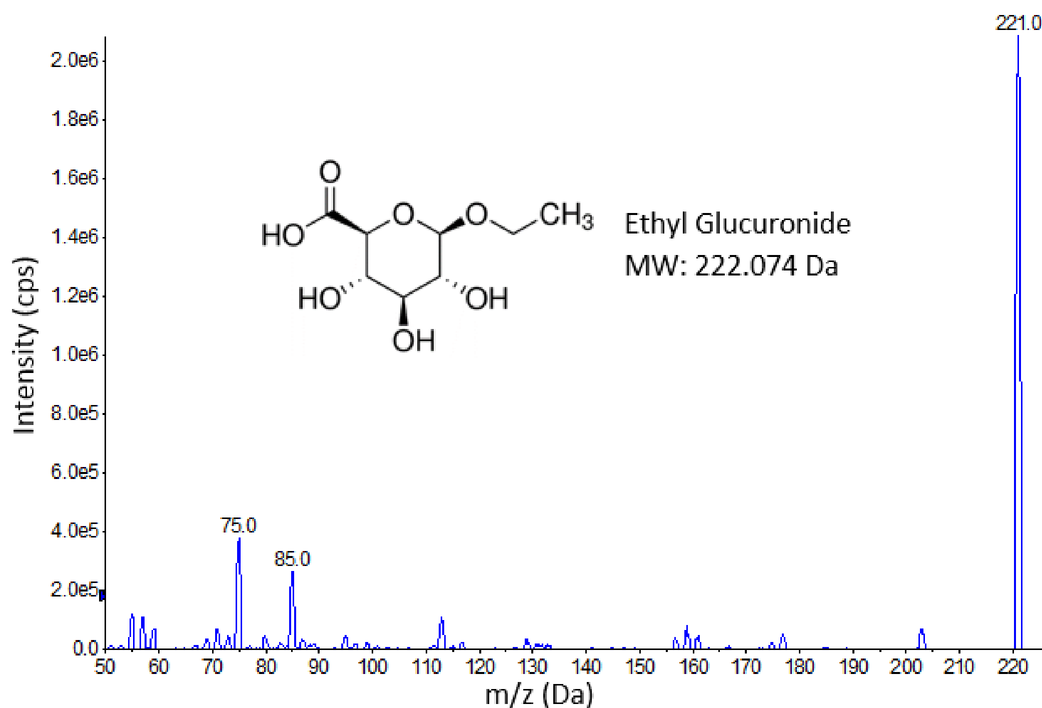

Figure 2.2 Initial product ion mass spectrum of ethyl glucuronide.

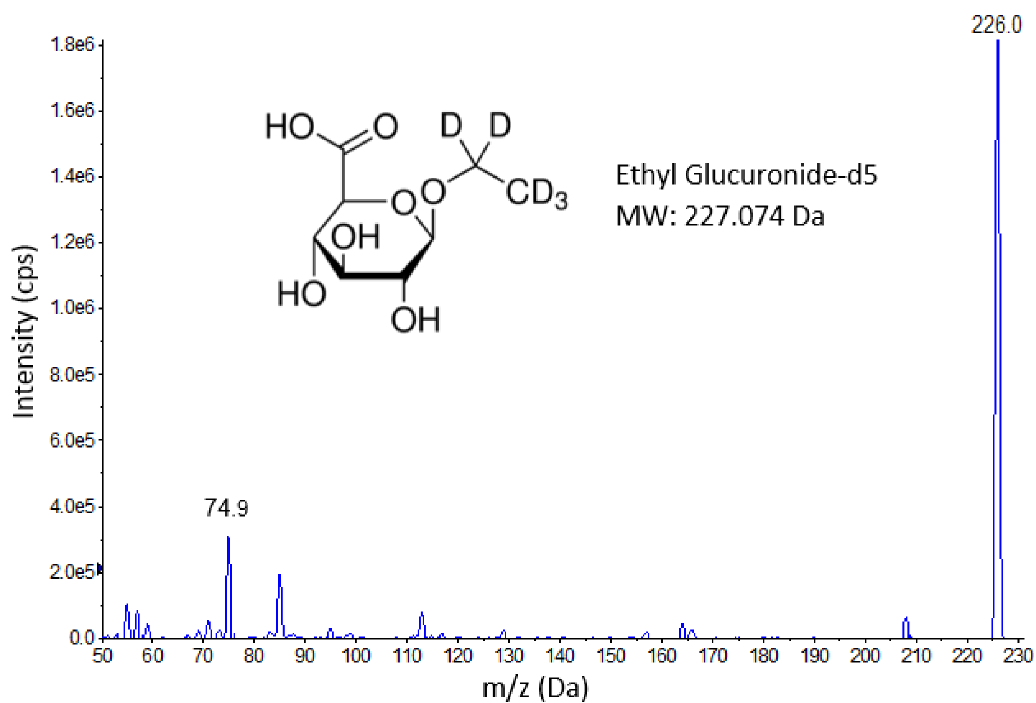

Figure 2.3 Initial product ion mass spectrum of ethyl glucuronide-d5.

Flow injection analysis was used to optimize the mass spectrometer conditions for EtG analysis. The ion spray voltage was set at -4500 V with a source temperature of 550°C. The nebulization, heating and curtain gases were set to 60, 70 and 30 psi respectively. The collision gas was nitrogen with the pressure set to medium. The MRM transitions and final mass spectrometer conditions are described in Table 2.1. Analyst software version 1.6.2 (SCIEX, Massachusetts, USA) was used to analyse the data collected.

Table 2.1 MRM transitions and final mass spectrometer conditions for the analysis of EtG and EtG-d5

Analyte	Transition	Dwell Time (ms)	Declustering Potential (V)	Entrance Potential (V)	Collision Energy (V)	Cell Exit Potential (V)
EtG	221 → 75.0	150	-120	-8	-20	-12
	221 → 85.0	150	-50	-10	-20	-10
EtG-d5	226 → 74.9	150	-30	-2	-20	-12

2.2.5 Method validation

2.2.5.1 Preparation of calibration standards and QCs

For the preparation of the calibration standards and QCs, EtG free hair was washed and pulverized according to methods described above. Pulverized hair (20 mg) was weighed into screw-cap tubes. Spiking solutions were prepared separately by spiking EtG into water. For the calibration standards EtG was spiked into water at a concentration of 32 ng/ml and serially diluted 1:1 with water to produce concentrations of 16, 8.0, 4.0, 2.0, 1.0 and 0.5 ng/ml (corresponding to the standards 480, 240, 120, 60, 30, 15 and 7.50 pg/mg).

For the preparation of the high, medium and low QCs as well as the LLOQ EtG was spiked into water at a concentration of 20 ng/ml (high QC) and diluted to produce concentrations of 15 ng/ml (medium QC), 1.25 ng/ml (low QC) and 0.5 ng/ml (LLOQ) (corresponding to high, medium, low QCs and the LLOQ at 450, 225, 18.8 and 7.50 pg/mg respectively). The concentration of internal standard used was 10 ng/ml (corresponding to 150 pg/mg).

Spiking solutions, with internal standard, for the calibration curve and QCs (300 µl) were added to the prepared aliquots of hair and extracted and analysed according to the methods described above. For each of the three validation batches the extracted calibration standards were analysed in duplicate and the QCs in six-fold. Calibration curves were constructed by plotting peak area ratio (peak area of the analyte peak/peak area of the internal standard peak) against analyte concentration.

Authentic hair, collected from a known heavy drinker, was washed and pulverized using the methods previously described. The hair was mixed well, to ensure that the sample was homogenous, and 20 mg aliquots were weighed into screw-cap tubes. These samples of hair were extracted and analysed as authentic QCs. Authentic QCs were included for analysis in each of the validation and sample batches to monitor the reproducibility of the extraction procedure.

2.2.5.2 Validation experiments

The method was validated according to the international guidelines of the Food and Drug Administration (FDA) [208, 209] as well as the European Medicines Evaluation Agency (EMEA) [210, 211]. The following validation experiments were performed: stock solution stability, accuracy and precision, matrix effects, specificity, sensitivity, carry-over, autosampler stability and long-term matrix stability.

2.2.6 Additional experiments

2.2.6.1 Extraction efficiency

To investigate the efficiency of the extraction method, five samples of authentic hair with known concentrations of EtG were washed according to the method previously described and subsequently extracted three times following the described extraction method. After the first extraction all of the extract was removed from the hair and the hair was left to air dry at room temperature before repeating the extraction process a further two times. The hair samples were only pulverized before the first extraction and this process was not repeated for the second and third extractions. The extracts from each of the extractions were filtered and analysed using the LC-MS/MS method described in order to compare the amount of EtG that remained in the hair after each of the extractions. The extraction procedure was performed in triplicate for each of the five hair samples.

2.2.6.2 Washout effect

The effect of washing hair with water, as in normal hygiene practises, on the potential loss of EtG from the hair was investigated using three samples of authentic, EtG positive, hair. Water (1 ml) was added to 20 mg aliquots of the three samples of authentic hair, in triplicate. The hair samples were gently shaken at 40 rpm for 15 min to simulate the normal hair washing procedure, although without the addition of shampoo. After 15 min the water from each of the hair samples was removed and transferred to separate eppendorf tubes. The hair samples were left to air dry at room temperature. This process was repeated a further two times. The collected water washes were filtered and analysed by the described LC-MS/MS method to determine the amount of EtG present, if any.

Additionally, aliquots of the three authentic hair samples that had not been exposed to the three water washes were washed, extracted and analysed according to the methods previously described. The amount of EtG in each of the water washes was compared to the amount of EtG extracted from these aliquots.

2.2.7 Analysis of study samples

The 257 study samples were quantitatively analysed using the validated method described above. Samples of hair (~ 20 mg) were cut from the proximal section of each of the collected samples with scissors, placed in 2 ml screw-cap tubes and weighed. The 0 – 3 cm segment was initially measured and weighed, however, for some of the samples there was too little hair in this segment and therefore the 0 – 6 cm segment was measured and weighed. The mass of the tube was subtracted from the total mass in order to get the exact mass of the hair sample, which was recorded along with the length of the segment of hair used. The average mass of hair weighed was 16.2 mg (±4.67). The hair samples were then washed, pulverized and extracted as described in Section 2.2.3.

2.2.8 Statistical analysis

GraphPad Prism 4 (California, USA) was used for the statistical analysis of data. Statistical significance was tested using the paired two-tailed t test (95% CI) and analysis of variance (ANOVA, 95% CI).

2.3 Results and Discussion

2.3.1 Method development and optimization

2.3.1.1 Wash method

There is no general consensus on the wash procedure that should be used when analysing hair for EtG. Additionally, there is no reference to a wash step in the guidelines suggested by the SoHT for the determination of EtG in hair [75]. A common wash procedure used in published methods involves ultrasonication with dichloromethane followed by a short methanol rinse step [81, 95–99].

In order to optimize the wash method, two wash procedures were compared using authentic, EtG positive, hair. These two wash procedures included a dichloromethane

wash followed by a methanol wash as described above and a wash procedure with only a dichloromethane wash, adapted from a method developed by Kintz et al. [111] Additionally, to measure any possible loss of EtG during washing, the wash solvents were analysed for EtG. The mean peak area ratio from each extracted hair sample was compared to the mean peak area ratio from the corresponding wash solvent.

The chromatograms produced after LC-MS/MS analysis of the wash solvents from the dichloromethane only wash method and the dichloromethane and methanol wash method appeared to be similar in that no peaks of significant size were observed in any of the chromatograms. Since EtG is a metabolite formed only after the consumption of alcohol, it is not possible for hair to become contaminated with external sources of EtG. Therefore, the main reason for washing hair before EtG analysis is not to remove external contamination, as is the case with some other drugs of abuse [56], but rather to remove impurities that might interfere with the analysis procedure. These results indicate that a single dichloromethane wash step is effective in doing so.

Morini et al. [99] reported interfering peaks at the EtG retention time as well as significant matrix effects when hair samples were washed only with methanol and not with dichloromethane. They concluded from this result that washing hair with dichloromethane is important in removing the lipid component of the hair matrix that resulted in the observed interferences. Additionally, the results from this current investigation showed that both solvents resulted in negligible amounts of EtG being lost during each of the wash procedures. Only 0.6% (±0.0135) of EtG was lost during the dichloromethane and 2.0% (±0.0511) during the methanol wash steps. These results are comparable to results reported in a similar study where 0.22% (±0.001) of EtG was lost during the dichloromethane and 2.3% (±0.224) during the methanol wash steps [99].

Based on these results it was concluded that a dichloromethane wash step alone is sufficient when analysing smaller samples of hair (20 – 30 mg) for EtG, as was done in this study. The solvent removed any lipid components that could have resulted in the occurrence of interfering peaks and did not result in significant amounts of EtG being

removed from the hair, as can be the case when using more polar solvents as wash solvents.

2.3.1.2 Extraction method

As with the wash procedure, there is no general consensus regarding the extraction procedure that should be used when analysing hair for EtG. The only recommendation made by the SoHT regarding the extraction procedure for EtG is that hair is pulverized into a powder before extraction [75]. During the development and optimization of the extraction procedure to be used for this study, the effect of pulverizing versus cutting samples of hair on the EtG recovery was investigated. Aliquots from four authentic hair samples (A, B, C and D), positive for EtG, were initially either cut into small pieces or pulverized into a fine powder prior to extraction. The amount of EtG extracted for each of the hair samples was compared, and the results are shown in Figure 2.4.

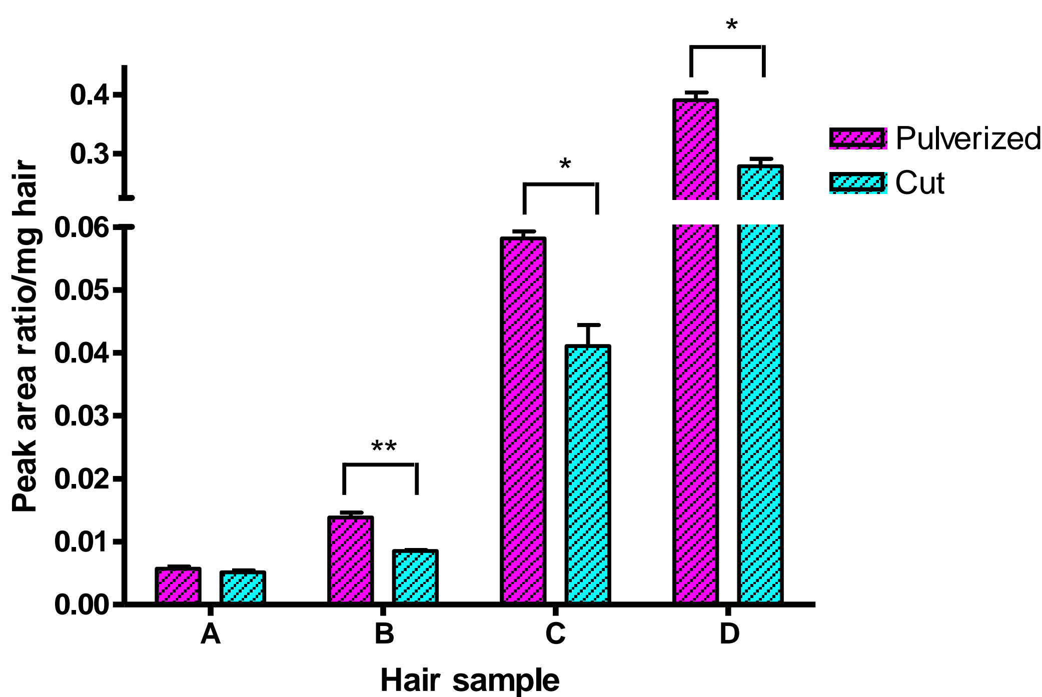

Figure 2.4 Comparison of the amount of ethyl glucuronide extracted from four authentic samples of hair after either pulverizing or cutting hair samples prior to extraction. Error bars show the standard deviation from the mean value (n = 3). Asterisks displayed on the graph show statistical significance between the two variables using a paired two-tailed t test at a 95% CI (* P<0.05; ** P<0.0055).

The results show that for all four authentic hair samples the amount of EtG extracted was greater when the hair had been pulverized prior to extraction. This is in agreement with results reported in previous studies [105–107]. For three of the four hair samples this increase was statistically significant. However, the effect of pulverization on EtG recovery was not consistent for the four hair samples and the percentage by which the amount of extracted EtG increased over non pulverized hair ranged from 10 to 40%. This variable effect of pulverization on the EtG recovery has been reported previously [204, 212]. In one study more than 200 hair samples analysed for EtG by either cutting or pulverizing the hair prior to extraction produced results with differences between the two methods ranging from – 41.7% to + 415% [204].

In an attempt to further optimize the extraction method the effect of an overnight incubation step and a 15 min compared to a 2 hour ultrasonication step was investigated using a sample of authentic, EtG positive, hair. The amount of EtG extracted for each of the different extraction conditions was evaluated by ANOVA (95% CI). The results of the statistical analysis showed no significant difference between the means of the EtG peak areas for each of the extraction conditions. Many published methods include an overnight incubation step [95, 97–99] and/or a 2 hour ultrasonication step [78, 93, 96, 111] resulting in a longer analysis time. Interestingly however, according to a recent multivariate analysis of the impact of several different extraction conditions on EtG recovery, the conditions that had the most significant influence on recovery were extraction solvent and incubation temperature [212]. In accordance with two earlier studies [109, 213], water is reported in this analysis to be the most effective extraction solvent. Despite most methods carrying out extractions at room temperature, results of the multivariate analysis suggest an incubation temperature of 60°C is better [212].

An extraction procedure with no overnight incubation and only a 15 min ultrasonication step has been previously reported, validated and successfully applied to 72 hair samples [214]. Pulverizing rather than cutting hair has also been shown to significantly reduce the incubation time needed [97]. Therefore, in light of the discussion above and the

results obtained, the extraction procedure for this current study was carried out at room temperature and simply involved adding water as the extraction solvent to pulverized hair followed by a short 15 min ultrasonication step.

2.3.1.3 Chromatography

The liquid chromatography method was optimized according to the type of column used, mobile phase selection and elution type (isocratic or gradient). The best retention of EtG was obtained using the Agilent Poroshell column (2.7 µm, 4.6 x 50 mm). Using this column the retention time of EtG was approximately 2 min with a total run time of 7 min. Comparison tests were done using a Waters Atlantis T3 column (3 µm, 2.1 x 100 mm) (Massachusetts, USA) designed for the retention of highly polar compounds such as EtG. However, the observed retention time was less than with the Poroshell column.

Most of the liquid chromatography methods that have been published for the analysis of EtG in hair make use of gradient chromatography with formic acid (0.1%) in water and acetonitrile, either with or without formic acid (0.1%), as the aqueous and organic mobile phases [93, 97, 105, 107, 110, 114, 212, 214]. During development and optimization of the current method the chromatography that resulted from an isocratic elution was compared to the chromatography achieved using a gradient elution. Additionally, the effects on the chromatography from using ammonium formate and ammonium acetate instead of formic acid in the aqueous mobile phase were also investigated. However, the best chromatography (based on analyte retention and peak shape) was produced when using a gradient elution with mobile phases 0.1% formic acid in water and 0.1% formic acid in acetonitrile. These results were to be expected considering the previously published methods discussed above. A representative chromatogram of the medium QC is shown in Figure 2.5.

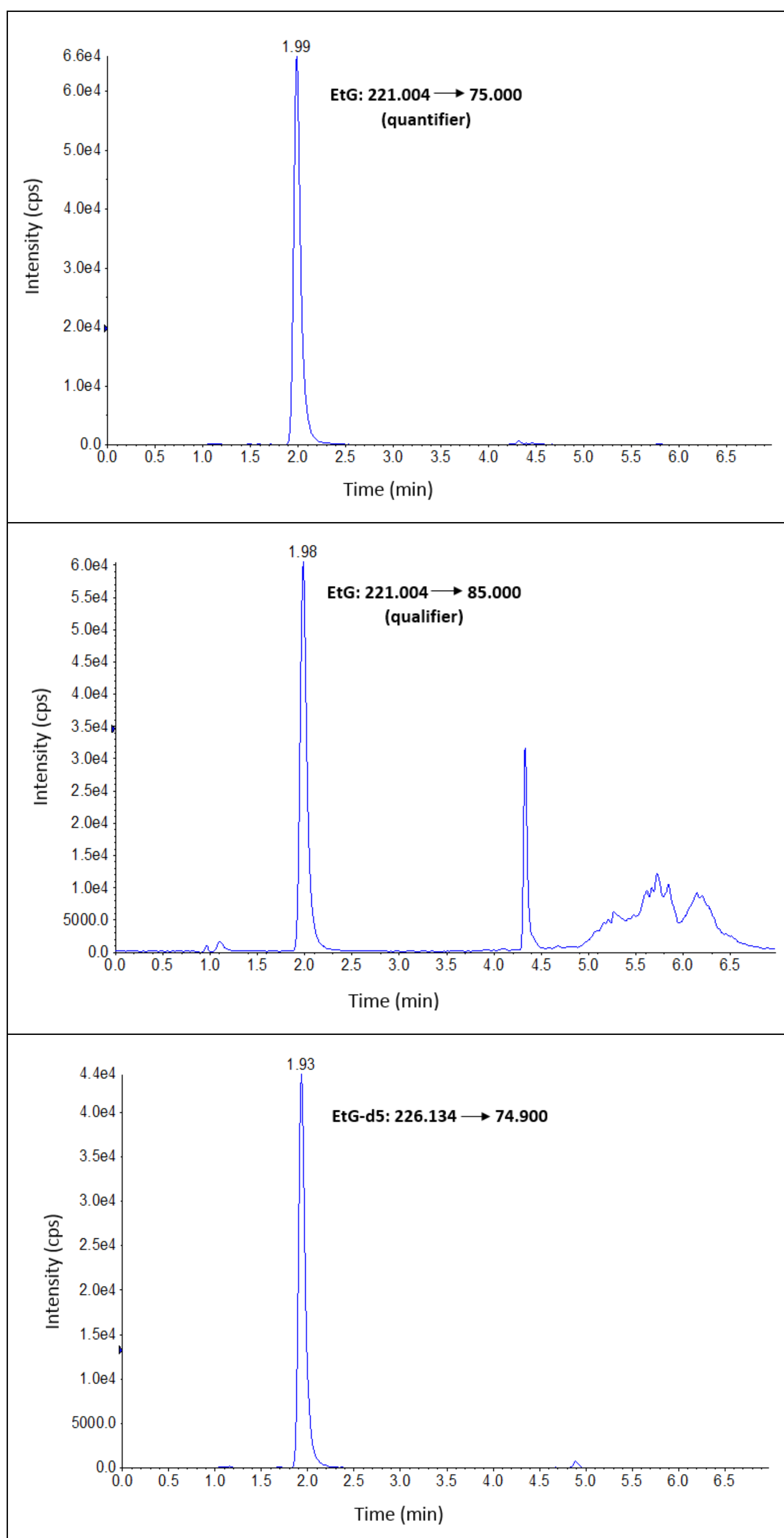

Figure 2.5 MRM chromatograms of blank hair spiked with ethyl glucuronide (225 pg/mg) and internal standard (150 pg/mg).

2.3.2 Method validation

2.3.2.1 Stock solution stability

To evaluate stability reference standard was diluted to 10 µg/ml with methanol and aliquots were stored at room temperature (~ 22°C), ~ 4°C and ~ -20°C for one week. For LC-MS/MS analysis 15 ng/ml dilutions of each of the stored aliquots and the reference standard, kept at ~ -80°C, were prepared in water in six-fold.

As shown in Table 2.2 the % difference between the average peak area ratio for the reference standard and the test solutions was less than 5%. The % CV was less than 2% for all of the measured values. According to international guidelines a % CV of greater than 15% and a difference of more than 15% could indicate instability in the stock solution [208, 210]. The results presented in Table 2.2 show that EtG is stable for up to one week when stored under the described conditions.

Table 2.2 Short-term stock solution stability of ethyl glucuronide when kept at room temperature (~ 22°C), ~ 4°C and ~ -20°C for one week

	Reference (~-80 ºC)	Test (room temp.)	Test (~ 4 ºC)	Test (~-20 ºC)
Peak area ratio 1	1.80	1.72	1.75	1.72
Peak area ratio 2	1.80	1.74	1.76	1.73
Peak area ratio 3	1.78	1.74	1.73	1.77
Peak area ratio 4	1.76	1.74	1.73	1.68
Peak area ratio 5	1.79	1.73	1.73	1.73
Peak area ratio 6	1.79	1.75	1.74	1.74
Average	1.79	1.74	1.74	1.73
STDEV	0.0151	0.0103	0.0126	0.0293
% CV	0.8	0.6	0.7	1.7
% Difference		**-2.8**	**-2.6**	**-3.3**

2.3.2.2 Calibration range

The method was validated over a calibration range of 7.50 – 480 pg/mg. This range includes the cutoff value of 30 pg/mg proposed by the SoHT for the identification of heavy drinking [75]. Calibration curves were generated for each of the three validation batches that were run. A quadratic regression equation was used weighted by 1/concentration. Each of the calibration curves showed a good fit, with all R values above 0.99. A representative calibration curve is shown in Figure 2.6.

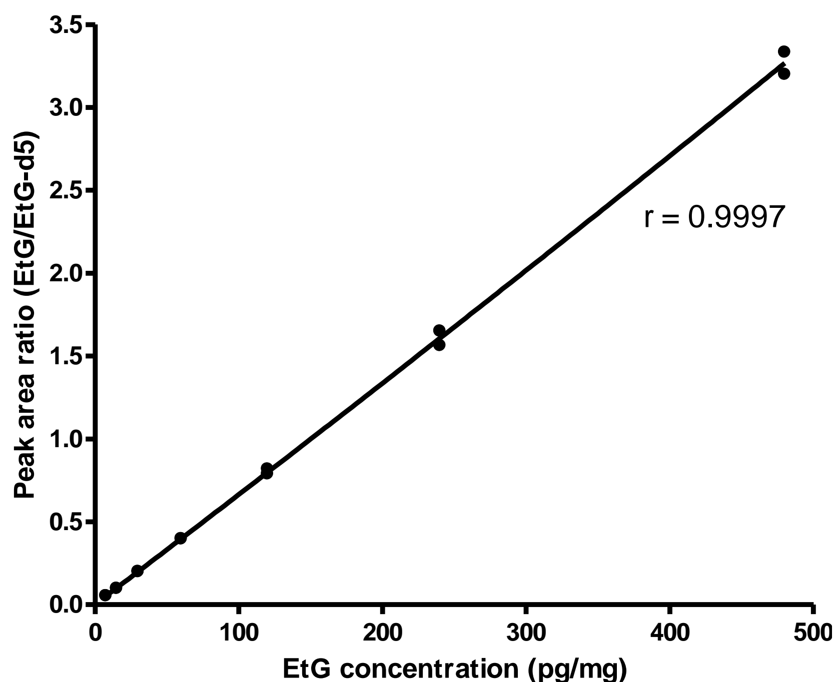

Figure 2.6 A representative calibration curve for ethyl glucuronide (Validation 3).

2.3.2.3 Accuracy and precision

The overall accuracy and precision of the validation process is determined by calculating the accuracy and precision statistics over the intraday and interday validation batches. Accuracy is expressed as the concentration of the analyte found as a percentage of the nominal concentration (% Accuracy) and precision is expressed as the coefficient of variation (% CV). For a valid method the intraday and interday accuracy is required to be within 15% over the entire calibration range and within 20% of the nominal concentration at the LLOQ. Additionally, the intraday and interday precision is required

to be less than 15% over the entire calibration range and less than 20% at the LLOQ [208]. The intraday and interday precision and accuracy results for the calibration standards are shown in Tables 2.3 and 2.4 respectively.

Table 2.3 Intraday accuracy and precision for ethyl glucuronide calibration standards

	Intraday						
	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7
Nominal concentration (pg/mg)	480	240	120	60.0	30.0	15.0	7.50
Average measured concentration (pg/mg)	480	240	121	59.9	30.0	14.5	7.74
n	2	2	2	2	2	2	2
STDEV	14.1	8.70	3.50	0.240	0.0445	0.122	0.156
% CV	2.9	3.6	2.9	0.4	0.1	0.8	2.0
% Accuracy	100.0	99.9	100.6	99.9	99.6	96.5	103.5

Table 2.4 Interday accuracy and precision for ethyl glucuronide calibration standards

	Interday						
	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7
Nominal concentration (pg/mg)	480	240	120	60.0	30.0	15.0	7.50
Average measured concentration (pg/mg)	480	240	120	60.0	29.7	14.9	7.58
n	6	6	6	6	6	6	6
STDEV	13.6	6.91	3.10	1.96	1.60	1.01	0.687
% CV	2.8	2.9	2.6	3.3	5.4	6.7	9.1
% Accuracy	99.9	100.0	100.3	99.9	99.1	99.7	101.1

Both the intraday and interday accuracy and precision for the calibration standards were found to be within acceptable limits according to FDA guidelines [208]. The intraday

% CV was below 4% and the % Accuracy between 96.5% and 103.5%. The interday % CV was below 10% and the % Accuracy between 99.1% and 101.1%.

The intraday and interday accuracy and precision results for the QCs, shown in Table 2.5, were also found to be within acceptable limits according to FDA guidelines [208].

Table 2.5 Intraday and interday accuracy and precision for ethyl glucuronide quality controls

	Intraday				Interday			
	QC High	QC Med	QC Low	LLOQ	QC High	QC Med	QC Low	LLOQ
Nominal concentration (pg/mg)	450	225	18.8	7.50	450	225	18.8	7.50
Average measured concentration (pg/mg)	445	225	18.6	7.66	438	224	19.3	7.9
n	6	6	6	6	18	18	18	18
STDEV	12.0	5.54	0.747	0.315	17.9	11.9	0.925	0.579
% CV	2.7	2.5	4	4.1	4.1	5.3	4.8	7.3
% Accuracy	99.3	99.8	99.1	102.0	97.3	98.9	102.5	106.5

The intraday % CV was below 5% and the % Accuracy between 99.1% and 102%. The interday % CV was below 8% and the % Accuracy between 97.3% and 106.5%. Even at the LLOQ, the % CV and % Accuracy fell well within the acceptable limits. From the validation results shown above for the calibration standards as well as the QCs, it can be concluded that the evaluated method is accurate and precise over the calibration range of 7.50 – 480 pg/mg.

In addition, six QCs at ten times the concentration of the QC high were also prepared and analysed within a validation batch. Before analysis, the extra high QCs were diluted 1:9 with blank extracted solvent. For these QCs the % CV was 4.2% and the % Accuracy 95.8%, both well within the acceptable limits. Therefore, samples with concentrations above the upper limit of the calibration curve can be diluted to within the calibration range with accuracy and precision.

The results from the analysis of the authentic QCs also showed good intraday and interday precision as shown in Table 2.6. The % CV's fell within the guidelines used to validate spiked QCs. The degree of homogeneity of authentic hair samples can influence the precision as the repeated extraction and analysis of a sample of hair that is not homogenous will result in poor precision due to the varying EtG concentrations in the respective aliquots of hair. Pulverizing hair has been shown to improve the homogeneity of hair samples and QCs prepared from authentic hair that has been pulverized have been shown to give better precision compared to QCs prepared from authentic hair that has been cut [215].

Table 2.6 Intraday and interday precision of authentic hair quality controls

	Intraday	**Interday**
Average measured concentration (pg/mg)	1465	1452
n	6	18
STDEV	85.6	90.9
% CV	5.8	6.3

The authentic QCs were prepared from hair that contained a high average concentration of EtG as seen in Table 2.6. A more accurate representation would have been to validate authentic QCs prepared over a range of concentrations, as was done in a previous study [102]. In the study hair from a known heavy drinker was diluted with hair collected from a teetotaller in order to obtain four authentic QC levels that were representative of the concentration range for sample analysis. Nonetheless, the results obtained from the analysis of the authentic QCs still validate the precision of the method. Unfortunately, since the real EtG concentration of authentic hair is unknown it is not possible to determine the accuracy of an extraction method using authentic QCs. Proficiency tests and QCs with a certified reference value are required in order to determine accuracy [107].

2.3.2.4 Matrix effects

The Matuszewski method [216, 217] was used to study possible ion enhancement or suppression effects that can be caused by components within the hair matrix. This method attempts to quantify the effect on the ionisation of the analyte across the calibration range. EtG blank hair obtained from eight teetotallers was spiked with EtG at high, medium and low QC concentrations and analysed in order to determine possible matrix effects. The results are shown in Table 2.7 below.

Table 2.7 Peak area ratios of high, medium and low quality controls to determine matrix effects for ethyl glucuronide in hair

	High 450 pg/mg	Medium 225 pg/mg	Low 18.8 pg/mg	Area Ratio vs Concentration Regression Slope
	Peak Area Ratio			
Matrix 1	3.70	1.83	0.124	0.00830
Matrix 2	3.89	1.86	0.124	0.00873
Matrix 3	3.79	1.92	0.133	0.00848
Matrix 4	3.78	1.86	0.137	0.00845
Matrix 5	3.75	1.86	0.136	0.00838
Matrix 6	3.61	1.76	0.118	0.00811
Matrix 7	3.80	1.79	0.143	0.00849
Matrix 8	3.87	1.90	0.156	0.00862
Average	3.77	1.85	0.134	0.00844
STDEV	0.0880	0.0533	0.0121	0.000190
% CV	2.3	2.9	9.0	**2.2**

The peak area ratios for each concentration level (high, medium and low) are used to generate regressions for each of the individual matrix sources. The slope variability (% CV) for the different matrix sources should not be greater than 5%. The slope variability for the eight different hair sources was 2.2%, indicating that matrix effects do not influence the precision of this method. Additionally, the deuterated internal standard used in this method compensates for any matrix effects that could be present. Therefore, despite the simple sample preparation procedure and the absence of a clean-up step, the above results show that matrix effects are limited.

2.3.2.5 Specificity

Specificity for EtG was evaluated by analysing blank hair samples collected from teetotallers (six in total) for possible interference between EtG and endogenous substances. An interfering peak at the EtG retention time was observed in some of the blank samples. The mean peak area of the interfering peaks, however, was less than 20% of the mean peak area observed when the same blank hair samples were spiked with EtG and analysed at the LLOQ. This result is acceptable according to international guidelines [208, 210]. The blank hair used for the preparation of the spiked calibration standards and QCs did not have an interfering peak at the EtG retention time. A representative blank chromatogram is shown in Figure 2.7.

Interfering peaks have been previously reported in literature [95, 105, 107]. Albermann et al. [105] optimized the chromatographic separation used in a previously published method [95] by using a 100% porous graphitic carbon column rather than a silica-based column. This allowed for better separation of the interfering peak away from the EtG retention time. Since the mean peak area of the interfering peaks observed in this method fell within acceptable criteria, it was not necessary to adjust the chromatography.

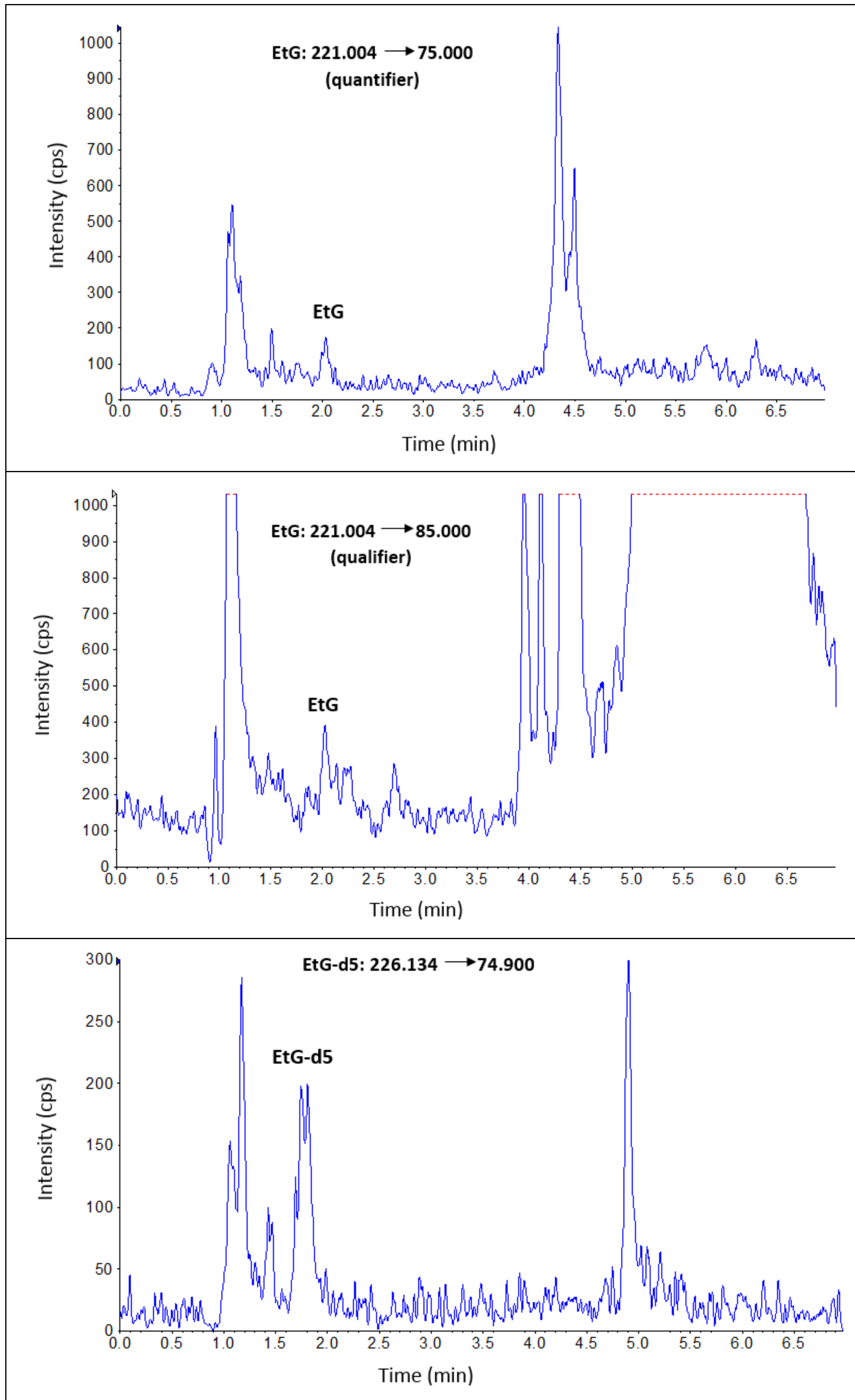

Figure 2.7 MRM chromatograms of a blank hair sample after ethyl glucuronide analysis.

2.3.2.6 Sensitivity

The LLOQ of the developed method is 7.50 pg/mg (Figure 2.7). FDA guidelines state that the mean analytes signal/noise (S/N) response at the LLOQ should be at least five times the response of a blank sample at the retention time of the analyte [208]. The S/N ratio of 23:1 for EtG, as shown in Figure 2.8, is acceptable and well exceeds the recommended S/N ratio of 5:1.

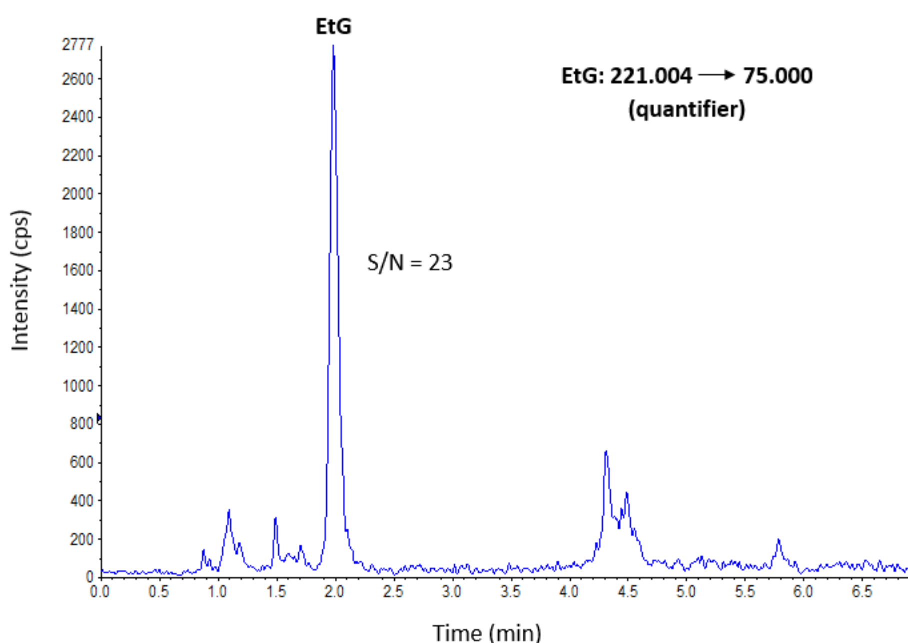

Figure 2.8 Chromatogram of blank hair spiked with ethyl glucuronide at the lower limit of quantification (7.50 pg/mg).

Previously developed LC-MS/MS methods report LLOQ values as low as 3 [100, 114] and 2 pg/mg [97] using 30 mg samples of hair. Binz et al. [218] validated a method using 20 mg of hair, with a reported LLOQ of 2.5 pg/mg. Although the sensitivity of this current method could have been improved to produce a lower value, the LLOQ of 7.50 pg/mg reported in this method far exceeds the cutoff value of 30 pg/mg proposed by the SoHT for the identification of heavy drinking [75]. Therefore, the LLOQ reported in this method is suitable for the purpose of this study to identify individuals who are heavy drinkers.

2.3.2.7 Carry-over

Carry-over was assessed and monitored during all of the analytical runs by injecting a double blank sample (containing no analyte or internal standard) immediately after the highest calibration standard. Additionally a blank sample (without analyte) was also included to determine any possible contamination of the analyte by the internal standard with an additional carry-over effect. Chromatograms of double blank and blank samples are presented in Figures 2.9 and 2.10.

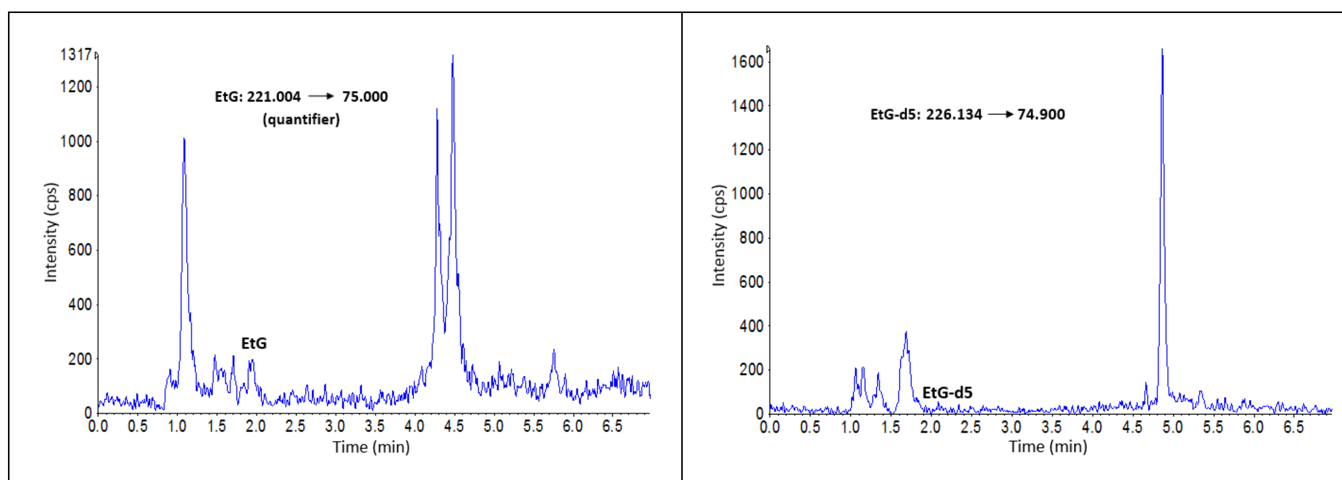

Figure 2.9 Chromatogram of a double blank hair sample. MRM transitions of the quantifying ion for ethyl glucuronide (left) and internal standard (right) are shown.

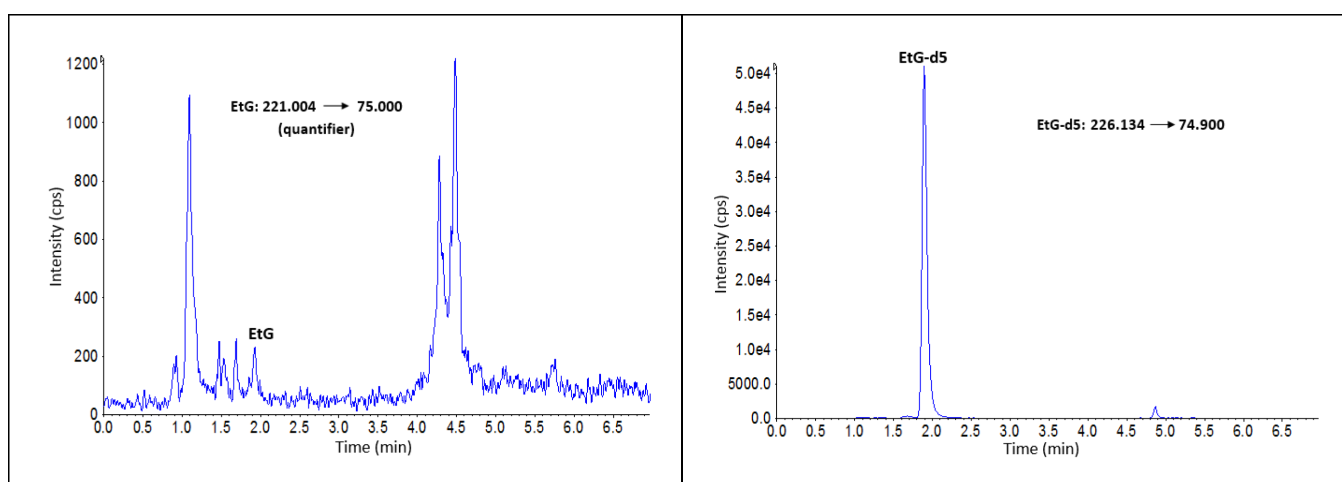

Figure 2.10 Chromatogram of a blank hair sample. MRM transitions of the quantifying ion for ethyl glucuronide (left) and internal standard (right) are shown.

The chromatogram of the double blank hair sample presented in Figure 2.9 shows that the double blank sample contains no significant carry-over of EtG from the previous injection of the highest calibration standard. Analyte carry-over is considered to be an issue when a peak that is observed for the analyte is greater than 20% of the peak area obtained at the LLOQ [210]. Similarly, the chromatogram of the blank hair sample presented in Figure 2.10 shows that there is no contamination of EtG by the internal standard and therefore there are no additional carry-over effects.

2.3.2.8 Autosampler stability

High and low QCs extracted in the first validation batch were left in the autosampler and re-injected after approximately 24 and 48 hours in order to evaluate the stability of EtG in processed samples. The peak area ratios of the re-injected QCs were compared to the peak area ratios of the QCs from the first injection. Comparisons of the peak area ratios for the re-injected high and low QCs with the QCs from the first injection are shown in Tables 2.8 and 2.9 respectively.

Table 2.8 Autosampler stability of extracted quality controls spiked at a high concentration of ethyl glucuronide

QC High (450 pg/mg)	Validation 1	Re-injection (~ 24 hours)	Re-injection (~ 48 hours)
	Peak Area Ratio		
Injection 1	2.91	2.76	3.19
Injection 2	3.10	2.78	3.20
Injection 3	3.18	2.81	3.26
Injection 4	3.18	2.84	3.24
Injection 5	3.14	2.84	3.26
Injection 6	3.19	2.89	3.25
Average	3.11	2.82	3.23
STDEV	0.105	0.0473	0.0320
% CV	3.4	1.7	1.0
% Difference after re-injection		**-9.5**	**3.8**

Table 2.9 Autosampler stability of extracted quality controls spiked at a low concentration of ethyl glucuronide

QC Low (18.8 pg/mg)	Validation 1	Re-injection (~ 24 hours)	Re-injection (~ 48 hours)
	Peak Area Ratio		
Injection 1	0.125	0.117	0.134
Injection 2	0.133	0.123	0.133
Injection 3	0.135	0.123	0.140
Injection 4	0.137	0.123	0.132
Injection 5	0.136	0.125	0.138
Injection 6	0.136	0.121	0.136
Average	0.134	0.122	0.136
STDEV	0.00431	0.00278	0.00287
% CV	3.2	2.3	2.1
% Difference after re-injection		**-8.5**	**1.5**

A high % CV and a % difference greater than 15% of the measured values indicates autosampler instability. The % CV was less than 3% for both the high and low QCs after 24 and 48 hour re-injections. Additionally, the % difference was less than 10% for both the high and low QCs after 24 and 48 hour re-injections.

However, these results are more relevant for samples of hair that have been spiked with EtG rather than authentic samples of EtG positive hair. Therefore, to determine the autosampler stability of authentic hair samples that have been processed for EtG, the above analysis was applied to authentic QCs that were also re-injected after 24 and 48 hours. Comparisons of the peak area ratios for the re-injected authentic QCs with the QCs from the first injection are shown in Table 2.10.

The % CV was less than 6% and the % difference less than 7% for the authentic QCs after 24 and 48 hour re-injections. This is a better representation of real hair samples and indicates that authentic samples processed for EtG are stable in the autosampler for up to 48 hours.

Table 2.10 Autosampler stability of authentic quality controls processed for ethyl glucuronide

Authentic QC	Validation 1	Re-injection (~ 24 hours)	Re-injection (~ 48 hours)
	Peak Area Ratio		
Injection 1	0.86	0.85	0.96
Injection 2	0.94	0.85	0.97
Injection 3	1.04	0.94	1.05
Injection 4	1.01	0.91	1.02
Injection 5	0.98	0.96	1.06
Average	0.96	0.90	1.01
STDEV	0.0678	0.0481	0.0472
% CV	7.0	5.3	4.7
% Difference after re-injection		**-6.3**	**4.8**

2.3.2.9 Long-term matrix stability

Authentic hair, from a known heavy drinker, was stored in the dark at room temperature for a period of nine months. Aliquots of hair (n = 6) were taken from the stored authentic hair, extracted and analysed for EtG at the time of storage, after one month, and again after nine months to evaluate the long-term stability of EtG in stored hair samples.

The % difference between the concentration of EtG measured after the initial analysis and the concentration measured after one and nine months was determined and is presented in Table 2.11. The results show a % difference of less than 5% after nine months indicating that EtG is stable in hair stored in the dark at room temperature for up to nine months.

Table 2.11 Long-term stability of ethyl glucuronide in authentic hair stored in the dark at room temperature

	Initial analysis	Analysis after one month	Analysis after nine months
	Concentration (pg/mg)		
Stability sample 1	1256	1467	1427
Stability sample 2	1281	1260	1569
Stability sample 3	1287	1437	1458
Stability sample 4	1759	1474	1497
Stability sample 5	1261	1365	1269
Stability sample 6	1343	1294	1353
Average	1365	1383	1429
STDEV	196	91.3	106
% CV	14.3	6.6	7.4
% Difference		**1.4**	**4.7**

One other research group has also evaluated the long-term stability of EtG in stored hair, although only for a period of four months [102]. Stability was determined in two samples of powdered hair, from known heavy drinkers, stored in the dark at room temperature and analysed repeatedly over a period of four months. Similar to the results above, the results showed that EtG was stable in the two samples of hair over a period of four months.

2.3.3 Additional experiments

2.3.3.1 Extraction reproducibility

A homogenous sample of authentic hair, from a known heavy drinker, was extracted and analysed on separate occasions over a period of four weeks. The purpose of this investigation was to evaluate the reproducibility of the extraction procedure when applied to authentic positive samples.

The results in Figure 2.11 show that the concentration of EtG being extracted from the authentic hair sample on numerous occasions over the period of four weeks was consistent and reproducible. A similar result was reported by Morini et al. [99] for which the concentration of EtG extracted from positive authentic samples of hair was reproducible when analysed over a period of five weeks.

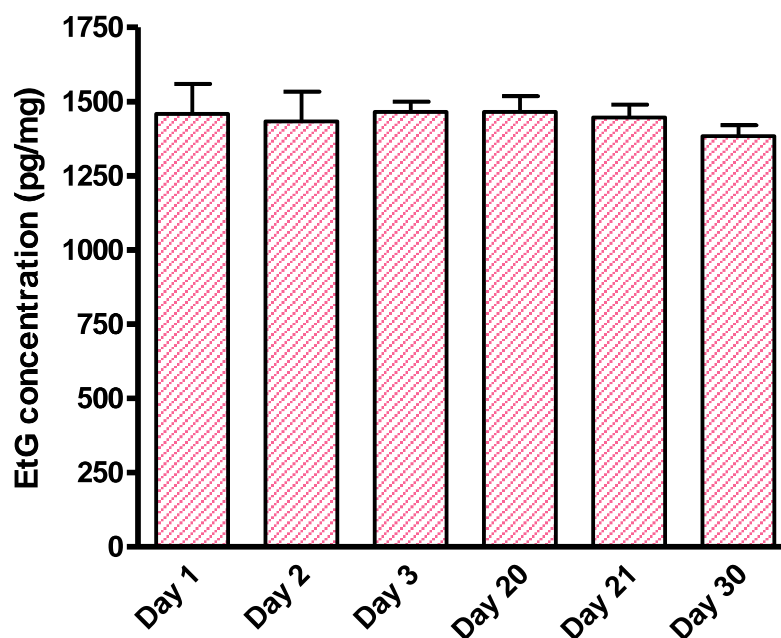

Figure 2.11 Concentration of ethyl glucuronide in an authentic sample of hair extracted and analysed on random days over a four week period. Error bars show the standard deviation from the mean value (n = 6). There is no statistical difference between the mean concentrations measured (ANOVA, 95% CI).

2.3.3.2 Extraction efficiency

Spiking blank hair with drugs or metabolites does not model the processes whereby drugs are incorporated into hair from the systemic circulation [219]. Therefore, determining the recovery using spiked hair may not be an accurate determination of the amount of drug actually extracted from hair. Using authentic hair samples to determine drug recovery is also difficult as the actual concentration of drug in the sample of hair is unknown.

A different approach to drug recovery was adopted by Huang et al. [197] who evaluated the extraction efficiency of their method by testing how much drug remained in the hair after being extracted. This method, however, was for the analysis of ARVs in hair and not EtG. This approach was adapted to determine the extraction efficiency of EtG from hair using the extraction method described in this study.

In order to determine the amount of EtG that remained in the hair after extraction, authentic hair samples were re-extracted an additional two times following the initial extraction. Five samples of hair from known alcohol consumers were used for this experiment. The EtG concentrations in the hair samples were 20 (sample G); 30 (sample H); 130 (sample E); 450 (sample F) and 1400 (sample D) pg/mg (determined previously using the validated method). These specific authentic hair samples were chosen so that the extraction efficiency for a range of EtG concentrations could be investigated.

The percentage EtG that was extracted for each of the three extractions was determined using the amount of EtG extracted after each extraction and the total amount of EtG extracted after all three extractions. The percentage EtG extracted after the first extraction ranged from 68% to 83% with an average of 77% as shown in Figure 2.12.

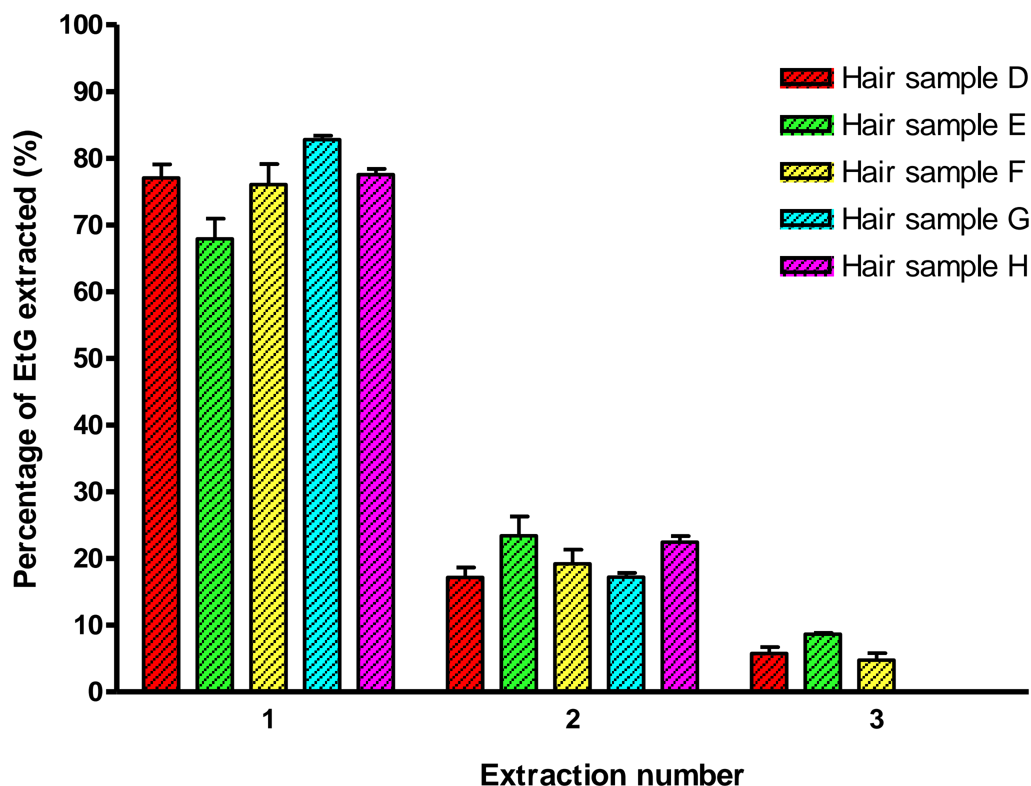

Figure 2.12 Comparison of the percentage of ethyl glucuronide extracted from five authentic hair samples after a series of three extractions, where the total amount of ethyl glucuronide extracted was calculated by adding the amount of ethyl glucuronide extracted after each of the three extractions. Error bars show the standard deviation from the mean value (n = 3).

The results also show that negligible amounts of EtG were extracted from hair samples G and H after the second extraction. These two samples had the lowest EtG concentrations (20 and 30 pg/mg) to begin with and were the samples from which the highest percentage of EtG was extracted after the first extraction. Less than 10% of the total EtG was extracted from samples D, E and F during the third extraction. These results indicate that the extraction method removed the majority of EtG from authentic samples of hair after the first extraction.

It is worthwhile to note the slight variation in the percentage of EtG extracted from the five different hair samples. This suggests that EtG is not uniformly extracted from different samples of hair that have different EtG concentrations. However, this is only a

preliminary result and would need to be repeated with a larger number of samples in order to confirm and further explore this observation.

2.3.3.3 Washout effect

As EtG is a very hydrophilic polar molecule, it is possible that normal hair hygiene could result in EtG being removed from the hair. This concept is referred to as the washout effect and has been previously reported and discussed in several publications [81, 91, 125, 206, 207]. To further explore this concept, three samples of authentic hair, from known alcohol consumers, were subjected to three successive water washes. After each wash step the water was removed from the hair sample and analysed for EtG. The amount of EtG in each of the water washes was compared to the amount of EtG extracted from aliquots of the same three samples of hair that had not been exposed to the three water washes.

The results of this experiment are represented in Figure 2.13, and show that EtG is removed when hair is in contact with water for a short period of time. The total percentage of EtG that was removed after all three water washes varied for the three hair samples. The most EtG was removed from hair sample A (64%). For hair samples B and D 36% and 45% of EtG was removed respectively. This result suggests that the hair sample itself may influence the extent to which EtG is removed from hair by water. It has been previously suggested that the condition of the hair, for example if it has been damaged by aggressive hair cosmetics, such as repeated bleaching, could be important regarding the extent to which EtG is removed from hair by the washout effect [81]. Unfortunately, no information regarding the use of cosmetic treatments was recorded and therefore the extent of the role played by the use of cosmetic treatments in the amounts of EtG that were removed from each of the three hair samples is unknown.

The results also show that the percentage of EtG that is removed from the hair decreases with each of the water washes. Only 2 to 3% of the total EtG was removed during the third water wash compared to 20 to 45% that was removed during the first water wash. This suggests that there might be a ‘limit’ to the amount of EtG that can be removed

from hair by water washes, after which complete disruption of the hair structure, through pulverization and/or ultrasonication, is necessary to further remove EtG incorporated into the hair shaft. This could potentially be linked to the processes by which EtG is incorporated into hair either through sweat deposition or through blood via the systemic circulation, however further investigation into this concept is necessary.

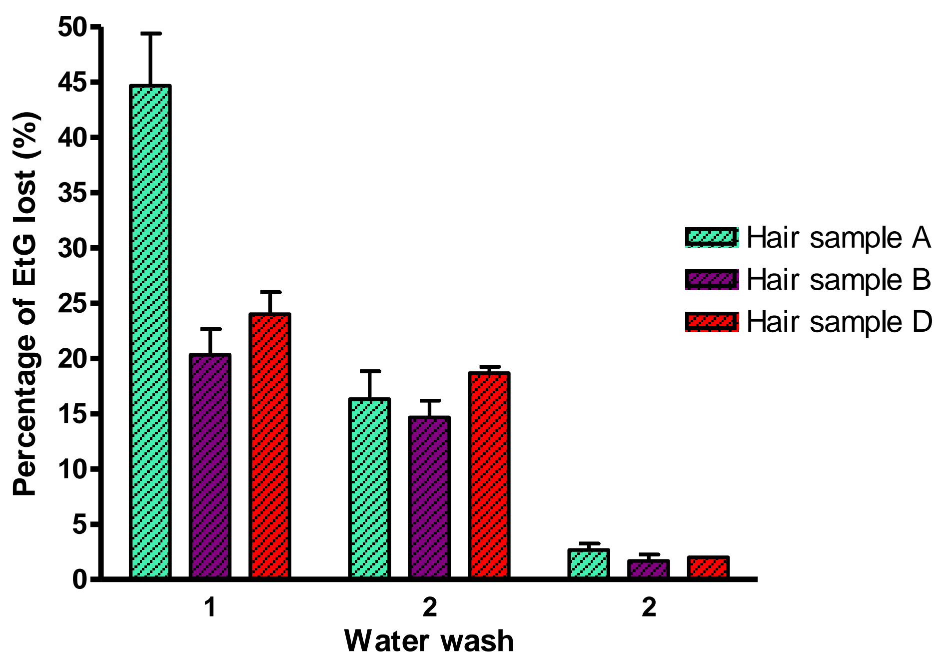

Figure 2.13 The percentage of ethyl glucuronide lost from three authentic hair samples after three successive water washes. Error bars show the standard deviation from the mean value (n = 3).

The experiment described here is a simple and novel approach to investigate the role that the washout effect plays in removing EtG from hair. The influence of shampoo on the washout effect was not investigated in this experiment. Although, it has been previously discussed that shampoo might be necessary to remove the greasy layer from hair before EtG can be removed [125, 213]. However, the results from this experiment indicate that water alone is sufficient for the removal of EtG from hair. Whilst these are only preliminary results they highlight the significant impact that the washout effect might have on the interpretation of EtG concentrations in hair, as has been discussed in literature [81, 206].

2.3.4 Analysis of study samples

The validated method for the quantification of EtG was used to analyse the samples of hair collected for the broader study on adherence in order to identify heavy drinkers. In total, 257 hair samples, collected from 135 individuals at 16, 32 and 48 weeks, were analysed. The EtG concentrations determined from the quantitative analysis of the hair samples are represented in Figures 2.14 and 2.15. The figures show the median concentration and interquartile range (IQR) for the social (Figure 2.14) and heavy (Figure 2.15) drinkers at weeks 16, 32 and 48. The cutoffs used in order to identify heavy and social drinking were EtG concentrations above 30 pg/mg, as suggested by the SoHT [75], and 7.50 pg/mg respectively.

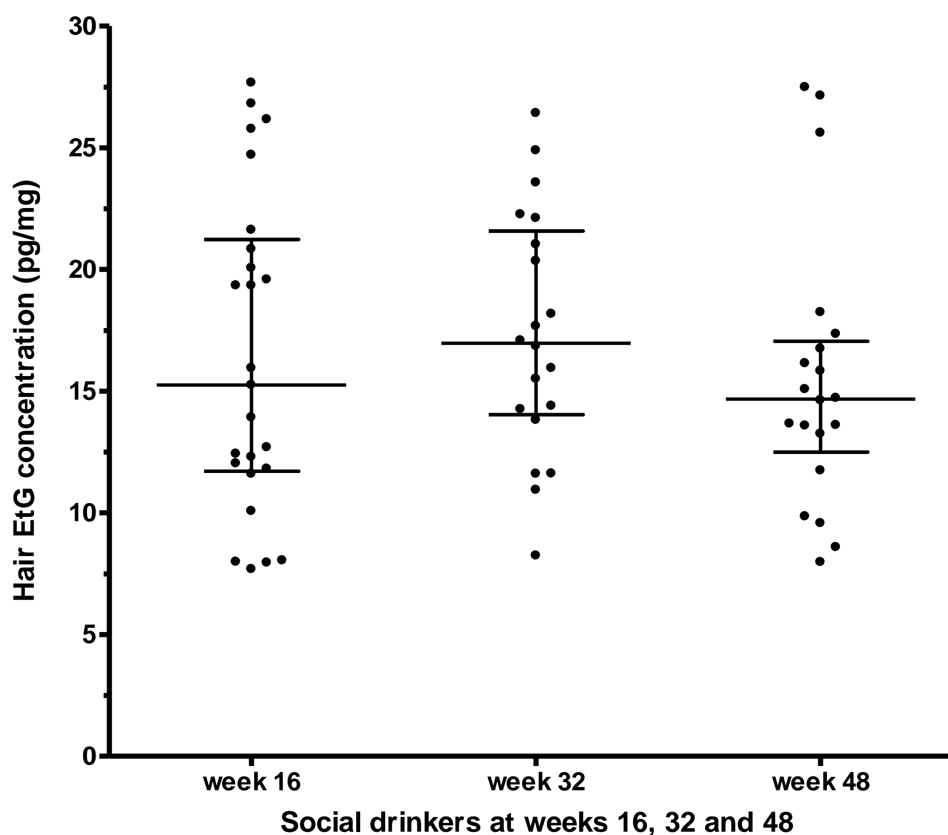

Figure 2.14 Median (interquartile range) ethyl glucuronide concentrations for social drinkers at weeks 16 (n = 25), 32 (n = 20) and 48 (n = 20).

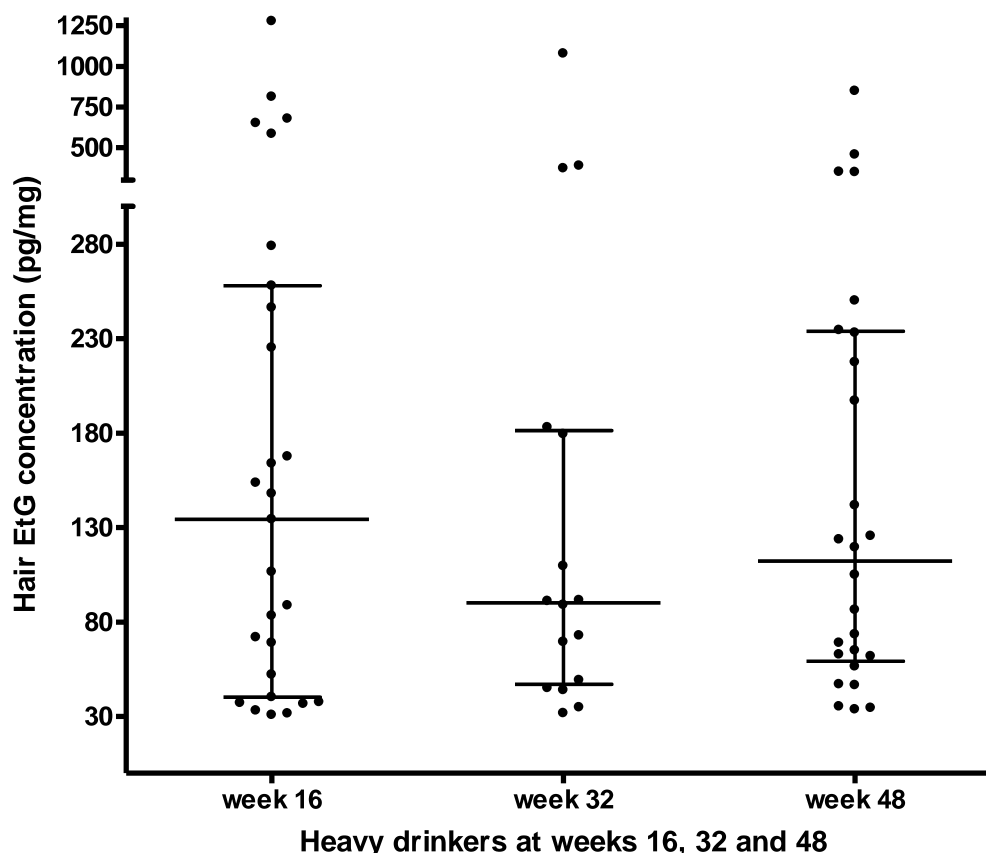

Figure 2.15 Median (interquartile range) ethyl glucuronide concentrations for heavy drinkers at weeks 16 (n = 27), 32 (n = 16) and 48 (n = 26).

Additionally, the results indicate that, overall, 27% of the samples contained concentrations of EtG above 30 pg/mg, 25% between 7.50 and 30 pg/mg and 48% below 7.50 pg/mg. The overall mean EtG concentration for the heavy drinkers was 201 pg/mg (±251), and 16.5 pg/mg (±5.76) for the social drinkers.

A representative chromatogram of one of the analysed study samples positive for EtG is shown in Figure 2.16.

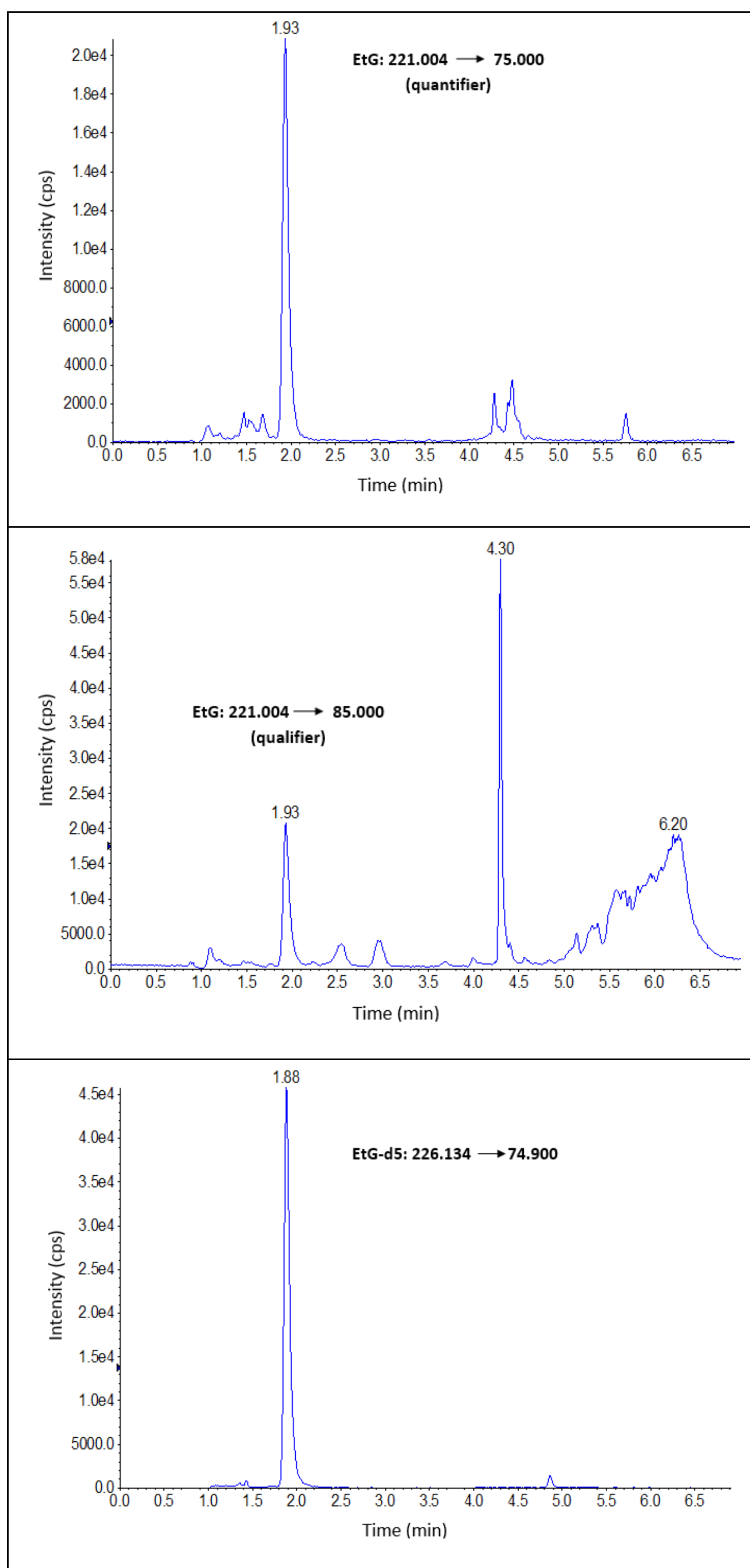

Figure 2.16 MRM chromatograms of an authentic hair sample positive for ethyl glucuronide (83.4 pg/mg), spiked with internal standard (150 pg/mg).

The results significant for the purposes of the broader adherence study are those with EtG concentrations above 30 pg/mg, as these results give an indication of the number of study participants who were abusing alcohol at the time of the study. However, it is interesting to note the range of EtG concentrations reported in general for the study population in order to gain an objective insight into drinking behaviour among HIV-infected patients. This is the first study, in South Africa, to assess drinking behaviour by quantitatively measuring the concentration of EtG in samples of hair. In one other study, hair samples for EtG and FAEE analysis were collected for a South African study, however, the samples were sent to an international laboratory for analysis [220]. Only 30 samples of hair were tested, and of these only two (7%) were positive for alcohol abuse based on FAEE analysis and 25% were positive based on EtG analysis.

There are, however, some limitations to the sample analysis performed in this current study. Firstly, despite the extraction method being validated for 20 mg of hair, the average mass of hair used for the analysis of the study samples was 16.2 mg (±4.67). Unfortunately, when the hair samples were collected it was unknown how much hair would be required for the analysis and in many cases insufficient amounts of hair were collected. To account for this, the concentration of EtG for each hair sample was calculated based on the amount of hair weighed for analysis. However, any correlation between EtG concentration and the mass of hair extracted for EtG was not evaluated.

Secondly, approximately 14% of the hair samples were shorter than the recommended 3 cm in length that could have resulted in EtG concentrations being misinterpreted. If hair samples are collected shortly after recent alcohol consumption the incorporation of EtG from sweat to the hair could cause an increase in the concentration of EtG in hair segments shorter than 3 cm in length [207]. Additionally, especially in the case of hair segments less than 1 cm in length, the hair segment only represents a short period of time and might not be sufficient to assess heavy drinking [81].

Lastly, no information regarding the use of cosmetic treatments was recorded and therefore it is unknown if the use thereof had any effect on the determined EtG concentrations or not. Hair samples were collected mainly from female participants and therefore information regarding cosmetic treatments would have been useful in interpreting the results.

According to the SoHT a concentration of EtG ≥ 7 pg/mg in hair suggests repeated alcohol consumption, and concentrations less than 7 pg/mg indicate abstinence [75]. However, the lowest concentration that this current method is able to measure accurately is 7.50 pg/mg, and therefore 7.50 pg/mg was used in the analysis of the study samples as the cutoff value to identify repeated alcohol consumption. As a result it is likely that there are individuals that have been misclassified as either social or non-drinkers, due to the lack of sensitivity of the method. However, the focus of the study was to identify heavy drinkers and therefore the lack of sensitivity was not a concern for the purposes of this study.

2.4 Summary and Conclusions

This chapter describes the development and validation of a method for the quantitative determination of EtG in hair. The method consists of a single dichloromethane wash step followed by a simple and quick extraction procedure using only 20 mg of hair. Hair samples were pulverized into a fine powder prior to extraction as results showed a mostly significant increase in the amount of EtG extracted from hair that had been pulverized compared to hair that was cut into small pieces.

The ability of the extraction procedure to effectively extract EtG from hair was investigated and the results showed that an average of 77% EtG was extracted from authentic hair samples after the first extraction. This result is based on the assumption that no EtG remained in the hair after the third extraction. For two of the samples

negligible amounts of EtG were extracted after the second extraction, however, for the other three samples a fourth extraction might have been necessary to remove any remaining EtG. Nonetheless, the results demonstrate that the extraction method was effective at removing most of the EtG from samples of hair in a short period of time. The fact that the results indicate that EtG is not uniformly extracted from different samples of hair suggests that the individual type of hair might also play a role in the extraction efficiency [212].

The method was successfully validated over the calibration range 7.50 – 480 pg/mg. The intraday and interday accuracy and precision results for both the calibration standards and QCs fell within accepted criteria demonstrating that the method was accurate and precise for the validated calibration range. Additionally, the intraday and interday precision determined from the analysis of authentic QCs fell within the acceptable limits used to validate spiked QCs. The results obtained from authentic QCs are valuable as they provide a more accurate representation of case samples compared to QCs that have been spiked.

Matrix effects were found to be within acceptable limits, despite the simple wash and extraction methods and absence of a clean-up step. The validated method, with the LLOQ set at 7.50 pg/mg, lacks the sensitivity of some previously published methods. Whilst the sensitivity of the method could have been improved to produce a more comparable LLOQ, the focus of this study was to rather develop a less time consuming extraction method, by excluding a clean-up step and using smaller amounts of hair; which ultimately limited the sensitivity of the method.

The washout effect seems to be a controversial topic regarding the analysis of hair for EtG and it is evident from literature that thoughts surrounding the role that this effect plays on the analysis of EtG in hair are mixed. Water is commonly used as the solvent for the extraction of EtG from hair during analysis, and therefore it is likely that EtG might be lost from hair during normal hair washing. The results from the simple experiment performed in this chapter indicate that up to 45% of EtG incorporated into

hair can be removed after exposure to water for 15 min. Even though this is only a preliminary result it is noteworthy, especially since water has been previously used to wash samples of hair prior to EtG extraction [78, 100–104]. Further research needs to be done in order to examine this effect in more detail so that the implications on EtG concentrations in hair can be determined.

Despite the limitations and uncertainties surrounding hair analysis for EtG, it is nevertheless a promising biomarker, highly sensitive and specific for the assessment of heavy drinking [121, 129, 221–223]. The validated method was used to analyse 257 authentic hair samples for EtG. Just over a quarter (27%) of the hair samples analysed contained concentrations of EtG above the 30 pg/mg cutoff proposed to identify heavy drinking. The implications of this in the context of the broader study on adherence will be discussed in Chapter 5.

This is the first study conducted completely within South Africa to analyse hair samples for EtG, and as such the first to provide objective insight into the drinking behaviour of HIV-infected patients in South Africa using hair analysis. As research into hair analysis for EtG develops in South Africa it will become necessary to determine the ideal cutoff values to be used within the South African context in order to accurately assess drinking behaviour.

3 Screening for drugs of abuse in hair

3.1 Introduction

In recent years, hair has become an important and valuable matrix for the analysis of drugs, either as an alternative or complementary matrix to blood and urine. One of the main advantages of hair analysis is that it provides a longer window (weeks, months or years) of drug detection and allows for retrospective investigation into past drug consumption [224]. Additionally, segmental analysis of hair is useful in providing a historic pattern of an individual's exposure to drugs [55]. The practical applications of hair analysis are widespread and include workplace drug testing, drug-related deaths, drug testing in the context of issuing and re-issuing drivers licenses, gestational drug exposure and drug-facilitated crimes [55, 62].

One of the challenges facing hair analysis is the correct interpretation of results. As such, hair analysis is limited by the possibility of providing false positive results caused by passive exposure to drugs; where an individual is exposed to but does not actively consume drugs. This is a common occurrence in the case of drugs that can be smoked such as AP, MP and COC [141, 225]. To minimise this problem hair samples are decontaminated by washing with various organic solvents and aqueous solutions before analysis [56]. However, wash procedures are not always sufficient to discriminate between passive exposure and active drug use [167].

This especially appears to be a concern with regards to the analysis of hair for COC, and studies have examined the effectiveness of various washing procedures at removing externally deposited COC from hair [152–154]. In addition to washing, the identification of specific COC metabolites can be used to distinguish COC use from contamination in some cases. The active COC metabolite, CE, is only formed in the human body when COC and alcohol are used at the same time [130, 131]. Therefore, the detection of CE indicates that COC must have been ingested and removes the question of external contamination [135].

Additionally, to further minimise reporting false positive results, the SoHT have recommended cutoff values for the most commonly abused drugs and their metabolites [56], and also recommend the use of metabolite-to-parent drug ratios [172]. The cutoff values recommended by the SoHT are based on literature pertaining to drug concentrations in hair determined from drug users to enable the identification of chronic drug use [56]. These cutoff levels were chosen to exclude drug levels potentially caused by environmental contamination as well as single drug use [62]. Research has shown that most drugs are present in the ng/mg range in the hair of regular drug users [141].

Due to the tendency of drug users to abuse more than one drug at a time (polydrug consumption), multi-analyte methods that are able to detect several compounds in a single run are useful [141]. Various analytical methods based on LC or GC techniques have been developed and validated over the years for the determination of drugs of abuse and their metabolites in hair. A thorough review of LC and GC based methods was recently published [141].

This chapter describes the validation of a qualitative LC-MS/MS method and application of the method to study samples in order to identify chronic drug use. Commonly abused drugs in South Africa include COC, MP (commonly known as *tik* to South Africans) and MQL (also referred to as mandrax) [226], and therefore these drugs of abuse, and their metabolites, were selected to be included in the screening method presented in this chapter. The recommended SoHT cutoff concentrations, given in Table 3.1, were used for the interpretation of results. No recommended cutoff concentration for MQL could be found and therefore 500 pg/mg was used. This is the same as the cutoff concentration recommended by the SoHT for COC and was decided upon to prevent false positive MQL results being reported. Additionally, no published hair analysis methods could be found that included MQL as one of the drugs of abuse being analysed.

Table 3.1 Drugs of abuse and metabolites included in the screening method with the corresponding Society of Hair Testing cutoff concentrations

Analyte	**Abbreviation**	**SoHT recommended cutoff concentration (pg/mg)** [56]
Amphetamine	AP	200
Methamphetamine	MP	200
Cocaine	COC	500
Benzoylecgonine	BZE	50
Cocaethylene	CE	50
Methaqualone	MQL	500*

* not recommended by the SoHT

Insufficient amounts of hair were collected from study participants which resulted in certain challenges to the study and specifically limited the screening of the hair samples for drugs of abuse. The limitations are discussed throughout the chapter, however, it is necessary to draw attention to the main limitation. Due to the limited amount of hair available, there was not enough hair left over for the extraction of the drugs of abuse. Therefore, the water extracts from the EtG extraction of the hair samples (presented in Chapter 2) were run through the drug screen. The polar extraction conditions used for the extraction of EtG from hair were most likely not favourable for the extraction of drugs of abuse and therefore brings uncertainty to the amount of drug actually extracted from the hair samples. Additionally, as the samples had already been extracted it was not possible to include any internal standard for the drugs of abuse. However, despite these and other limitations, the screening method was successfully used to identify chronic drug use within the study population.

3.2 Materials and Methods

3.2.1 Collection and storage of hair samples

3.2.1.1 Blank samples

Drug free hair, collected from a child, was used for the preparation of cutoff QCs and in validation experiments. Nine additional sources of drug free hair were collected from adult volunteers, with no history of drug use, for matrix effects and specificity experiments. A hair repository was registered with the University of Cape Town, Faculty of Health Sciences, Human Research Ethics Committee for the collection and storage of hair samples (reference R026/2015). Volunteers provided verbal consent to their hair being collected. All hair samples registered in the repository are stored in paper envelopes at room temperature.

3.2.1.2 Study samples

Chapter 2, Section 2.2.1.2, provides details regarding the hair samples collected for the broader adherence study.

3.2.2 Chemicals and reagents

The following reference standards were purchased from Cerilliant (Texas, USA) as solutions at concentrations of 1 mg/ml in methanol: BZE and MQL, and in acetonitrile: COC and CE. AP and MP were purchased from Cerilliant (Texas, USA) as solutions within a mixture of six amines at a concentration of 250 µg/ml in methanol. Working solutions were prepared by diluting each of the reference standards to 10, 1 µg/ml and 100 ng/ml with either methanol or acetonitrile. All reference and working solutions were stored below -20°C. Methanol and acetonitrile (LC-MS grade) were purchased from Honeywell International Inc. (Mexico City, Mexico). Ammonium Formate was purchased from Sigma-Aldrich (Missouri, USA). Deionized water was prepared using a Synergy Water Purification System purchased from Merck Millipore (Massachusetts, USA).

3.2.3 LC-MS/MS Conditions

The LC-MS/MS method currently used in the Division of Clinical Pharmacology, Department of Medicine, University of Cape Town, for the screening of forensic toxicology samples (mainly blood and urine) for drugs of abuse was used in this screening method. The LC-MS/MS conditions are described below.

An Agilent Technologies 1260 Infinity Liquid Chromatography system (California, USA) coupled to an AB SCIEX Q Trap 5500 mass spectrometer (SCIEX, Massachusetts, USA) was used for the analysis of the six analytes included in the screening method. Chromatographic separation was achieved using a Kinetex EVO C18 column (5 µm, 3 x 50 mm) (Phenomenex, USA) kept at 40°C. Ammonium Formate (10 mM, pH 6.5) was used as mobile phase A and a mixture of methanol and acetonitrile (50:50, v/v) as mobile phase B. The system was run using a gradient program starting at 5% phase B increased to 98% over 6 min. At 6.1 min phase B was decreased back to 5% where it was held for 4 min. The total run time was 10 min at a constant flow of 500 µl/min.

The mass spectrometer was set to electrospray ionization in the positive MRM mode and each of the analytes were monitored using a single qualifying transition. The ion spray voltage was set at 5500 V with a source temperature of 500°C. The nebulization, heating and curtain gases were set to 65, 45 and 30 psi respectively. The dwell time was set to 80 ms for each of the analytes. The MRM transitions, retention times and final mass spectrometer conditions are described in Table 3.2. Analyst software version 1.6.2 (SCIEX, Massachusetts, USA) was used to analyse the data collected.

Table 3.2 Overview of the LC-MS/MS parameters for the selected drugs of abuse and metabolites

Analyte	Transition	Retention Time (min)	Declustering Potential (V)	Entrance Potential (V)	Collision Energy (V)	Cell Exit Potential (V)
AP	136 → 119	3.07	51	10	7	12
MP	150 → 119	3.48	51	10	15	12
COC	304 → 182	4.42	46	10	27	12
BZE	290 → 168	3.54	111	10	25	12
CE	318 → 196	4.77	91	10	27	12
MQL	251 → 132	5.37	80	10	45	12

3.2.4 Method validation

3.2.4.1 Preparation of the cutoff QC

For the preparation of cutoff QCs drug free hair was washed and pulverized according to the methods described in Chapter 2, Section 2.2.3, so that the QCs were prepared in the same way as the study samples. Briefly, hair was washed with dichloromethane and ultrasonicated for 5 min. Washed hair samples (20 mg) were pulverized into a fine powder using the Omni Bead Ruptor 24 (Omni International Inc., Georgia, USA) at a speed of 6 m.s^{-1} for four cycles of 60 s each with a dwell time of 30 s after each cycle.

The QC working solution, used to spike drug free samples of hair, was prepared by spiking each of the six analytes into water at the respective cutoff concentrations, provided in Table 3.1. The QC solution was aliquoted and kept at ~ -20°C until needed. QC solution (300 µl) was added to the prepared aliquots of hair and extracted according to the method previously developed for the extraction of EtG (Chapter 2, Section 2.2.3.2). Briefly, after adding the solution, the samples were vortex mixed for 10 s, ultrasonicated for 15 min and the extract filtered through a 0.45 µm filter under vacuum (Merck Millipore, Massachusetts, USA). The clear extract (2 µl) was injected into the LC-

MS/MS system for analysis. To determine the precision of the assay, the cutoff QC was prepared, extracted and analysed in six-fold for each of the three validation batches.

3.2.4.2 Validation experiments

The FDA [208, 209] and EMEA [210, 211] validation guidelines referred to previously in Chapter 2 are for the validation of quantitative methods and do not describe the criteria necessary for the validation of qualitative methods. The Scientific Working Group for Forensic Toxicology (SWGTOX) published guidelines in 2013 for method validation in forensic toxicology [227]. These guidelines recommend the evaluation of the following parameters when validating qualitative methods: carry-over, interference studies, ionization suppression/enhancement, LOD and stability. It has also been suggested in the literature that the validation of qualitative methods include the evaluation of the following validation parameters: specificity, LOD, extraction recovery and intraday precision [228–230]. Therefore, to ensure the reliability of the results produced and in accordance with the recommendations and suggestions mentioned above, the validation of the qualitative method included an evaluation of the following parameters: accuracy and stability of the QC working solution, precision, matrix effects, specificity and stability of an extracted QC. Unless otherwise stated the validation criteria according to the FDA and EMEA guidelines were applied where applicable.

Accuracy and stability of the quality control working solution

To determine the accuracy of the QC working solution, two independent working solutions (WS1 and WS2) were prepared by two different analysts on the same day and analysed using the LC-MS/MS method previously described. The average peak areas for each of the six analytes in WS1 and WS2 were compared in order to determine accuracy. To determine the stability of the QC working solution, a working solution was prepared and stored at ~ -20°C for two weeks. After two weeks the stored QC solution was tested against a freshly prepared reference QC solution, and the average peak areas for each of the six analytes compared in order to determine stability.

Matrix effects

As only one QC level was included in this screening method the Matuszewski method [216, 217] could not be applied to study possible ion enhancement or suppression effects. Instead, to get an indication of matrix effects in the absence of any internal standard, ten different samples of drug free hair were spiked with the cutoff QC, extracted and analysed using the methods previously described. The variation in the peak areas (% CV) for each of the six analytes in the ten different hair samples was determined.

Specificity

Specificity for the six analytes was evaluated by analysing ten different samples of drug free hair for possible interferences between the analytes of interest and endogenous substances. The hair samples were extracted and analysed according to the described method. According to international guidelines the peak area of a peak present at the analytes retention time should be less than 20% of the peak area at the LLOQ of the analyte [208, 210]. For the purposes of this study any peaks present at the retention times of interest with a peak area of less than 20% of the respective peak area in the cutoff QC were considered acceptable.

Stability of quality control extract

Stability of the extracted analytes was determined by spiking QC working solution (300 µl) into washed and pulverized drug free hair (20 mg). QCs were extracted according to the method developed for the extraction of EtG from hair (Chapter 2, Section 2.2.3) briefly described above and stored at ~ -80°C for four months. After four months the stored QC extract was tested against a QC extract prepared on the day of analysis. The average peak areas for each of the six analytes in both the stored and fresh QC extracts were compared in order to determine stability.

3.2.5 Analysis of study samples

Due to the limited amount of hair collected there was insufficient amounts of hair available after the analysis of the 257 study samples for EtG (Chapter 2) to extract the hair for drugs of abuse. Therefore, after the samples had been processed and analysed for EtG, the water extracts were frozen, in the original 96-well plates, at ~ -80°C for four months until screening. On the day of screening, the respective plates were removed from the freezer and the extracts allowed to thaw at room temperature. Thereafter, 2 µl of the thawed extract was injected into the LC-MS/MS system for analysis.

3.3 Results and Discussion

3.3.1 Chromatography

The analytes were well retained on the Kinetex EVO C18 column, with all six analytes eluting during the period of time in which the gradient increased from 5% to 98% mobile phase B. A representative chromatogram is presented in Figure 3.1. Although slight peak tailing was observed for the analytes AP and MP, the chromatography was considered to be satisfactory for the qualitative purpose of this method.

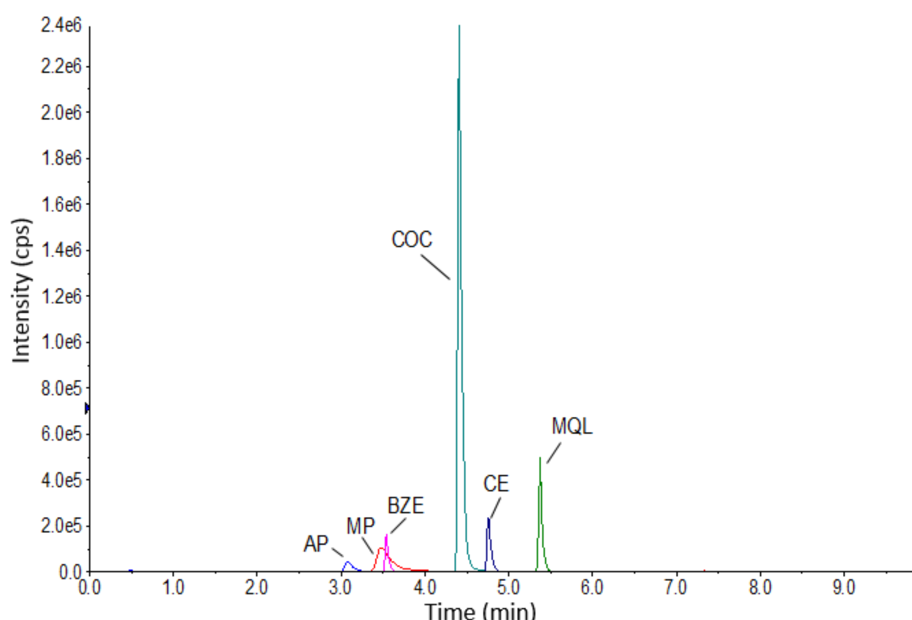

Figure 3.1 Representative MRM chromatogram of the cutoff quality control sample in hair. The peaks of the single qualifying transitions for each of the analytes are shown.

3.3.2 Method validation

3.3.2.1 Accuracy and stability of quality control working solution

As the screening method contained only one QC, prepared at the respective cutoff concentrations for each of the six analytes, it was important to validate this QC with regards to the accuracy of the preparation of the solution and the respective concentrations of each of the analytes. The % difference between the average peak areas for each of the analytes in the two independent QC solutions, WS1 and WS2, are presented in Table 3.3.

Table 3.3 Accuracy of cutoff quality control working solutions 1 and 2

Analyte	WS1			WS2			% Difference
	Average peak area	STDEV	% CV	Average peak area	STDEV	% CV	
AP	175667	4633	2.6	176833	3488	2.0	**0.7**
MP	865333	15731	1.8	881000	28121	3.2	**1.8**
COC	5911667	34881	0.6	6155000	83126	1.4	**4.1**
BZE	258000	3347	1.3	271500	2510	0.9	**5.2**
CE	583833	9806	1.7	619833	8886	1.4	**6.2**
MQL	1050000	12649	1.2	974000	11225	1.2	**-7.2**
n	6			6			

International guidelines for the validation of quantitative methods state that for the solutions to be accurate, the % difference between the two solutions should not be greater than 5% [208, 210]. According to this criteria the % difference for the analytes BZE, CE and MQL (5.2%, 6.2% and -7.2% respectively) should not be acceptable. However, as this method is qualitative and for screening purposes only, these results were accepted and the two solutions considered to be sufficiently accurate.

QC working solutions were prepared in water and kept at ~ -20°C until needed for the duration of the sample screening process. It was therefore necessary to determine the stability of the analytes in the stored QCs under these conditions. As shown in Table 3.4, the % difference between the average peak areas for the fresh and stored QC working

solutions was less than 15% for each of the six analytes tested. These results are within international guidelines requirement of a % difference of less than 15% to demonstrate stability [208, 210]. Therefore, QC solutions were prepared and kept at ~ -20°C for up to two weeks.

Table 3.4 Stability of analytes within the quality control working solution kept at ~ -20°C for two weeks

Analyte	Fresh WS			Stored WS (~ -20°C)			% Difference
	Average peak area	STDEV	% CV	Average peak area	STDEV	% CV	
AP	410833	3601	0.9	371500	9915	2.7	**-9.6**
MP	1751667	7528	0.4	1593333	38816	2.4	**-9.0**
COC	9653333	166693	1.7	9928333	70261	0.7	**2.8**
BZE	523333	8959	1.7	449333	8802	2.0	**-14.1**
CE	1002833	10206	1.0	1071667	16021	1.5	**6.9**
MQL	2081667	21370	1.0	2196667	18619	0.8	**5.5**
n	6			6			

3.3.2.2 Quality control precision

The intraday and interday precision of the cutoff QC was determined in order to assess the reproducibility of the assay. The results presented in Table 3.5 show that, for all of the analytes, the intraday % CV was below 4% and the interday % CV was below 14%. The precision of the assay therefore meets international criteria recommended for the validation of QCs in a quantitative method.

As a result of the absence of any internal standard, the interday % CV observed for each of the analytes was greater than the intraday % CV. This was most likely caused by day to day variability in the LC-MS/MS system which an internal standard would usually correct for, resulting in less variation when comparing results from samples analysed on different days or in different batches. Nevertheless, even without an internal standard, the results shown in Table 3.5 are acceptable and demonstrate the precision of the assay.

Table 3.5 Intraday and interday precision for the drugs of abuse and metabolites in the cutoff quality control

Analyte	Precision (% CV)	
	Intraday	Interday
AP	3.2	13.1
MP	1.6	12.0
COC	1.9	8.8
BZE	2.7	9.5
CE	1.8	9.9
MQL	2.9	10.0
n	6	18

Unfortunately, no authentic drug positive hair was available for this study and as such authentic QCs could not be included in the validation of the method. As discussed in Chapter 2, the process of spiking hair with drugs does not reflect the processes by which drugs are incorporated into the hair matrix [219]. Therefore, the inclusion of authentic QCs, where possible, allows for a more accurate representation of the reproducibility of the assay.

3.3.2.3 Matrix effects

The peak areas for each of the analytes extracted from the ten different samples of drug free hair spiked with the cutoff QC, as well as the % CVs are shown in Table 3.6. For the analytes MP, BZE and MQL the variation in the peak areas measured in the different hair samples was less than 15% and as such matrix effects were not considered to significantly affect the ionization of these analytes at the respective cutoff concentrations. However, the % CV for AP, COC and CE (19.2%, 24.3% and 31.1% respectively) was greater than 15% indicating that the ionization of these analytes was more likely to be affected by components within the hair matrix. Therefore, positive results for these analytes should be interpreted with caution, especially in the case of CE which had the highest % CV of 31.1%.

Table 3.6 Peak areas for the drugs of abuse and metabolites in ten different drug free samples of hair spiked at the cutoff concentrations and extracted to determine matrix effects

	AP 200 pg/mg	MP 200 pg/mg	COC 500 pg/mg	BZE 50 pg/mg	CE 50 pg/mg	MQL 500 pg/mg
	Peak area					
Matrix 1	217000	885000	6040000	353000	591000	946000
Matrix 2	152000	709000	4340000	491000	343000	767000
Matrix 3	200000	808000	5370000	380000	528000	775000
Matrix 4	160000	681000	3050000	338000	290000	678000
Matrix 5	155000	700000	4430000	355000	401000	692000
Matrix 6	200000	778000	6060000	358000	619000	930000
Matrix 7	170000	709000	5120000	357000	492000	932000
Matrix 8	141000	636000	3530000	361000	289000	802000
Matrix 9	108000	511000	3080000	406000	245000	943000
Matrix 10	176000	761000	5170000	351000	475000	745000
Average	167900	717800	4619000	375000	427300	821000
STDEV	32206	101580	1122700	44821	132982	106963
% CV	19.2	14.2	24.3	12.0	31.1	13.0

3.3.2.4 Specificity

A representative chromatogram produced after the extraction and screening of a drug free sample of hair is shown in Figure 3.2. No interfering peaks were observed in any of the drug free hair samples for four of the six analytes (namely AP, COC, BZE and CE). However, in all the drug free samples of hair that were extracted and analysed, peaks were observed at the same retention time as MP and MQL. These peaks were observed, at the same intensity, in blank water injections as well. Therefore, it was determined that the peaks were not due to interferences caused by endogenous substances, but rather a result of system carry-over. The peak areas were less than 20% of the peak areas of the MP and MQL peaks in the cutoff QC, and are therefore not a concern for the purposes of this study. However, if a more sensitive screening method is required in the future, the carry-over observed could become a problem and will need to be addressed.

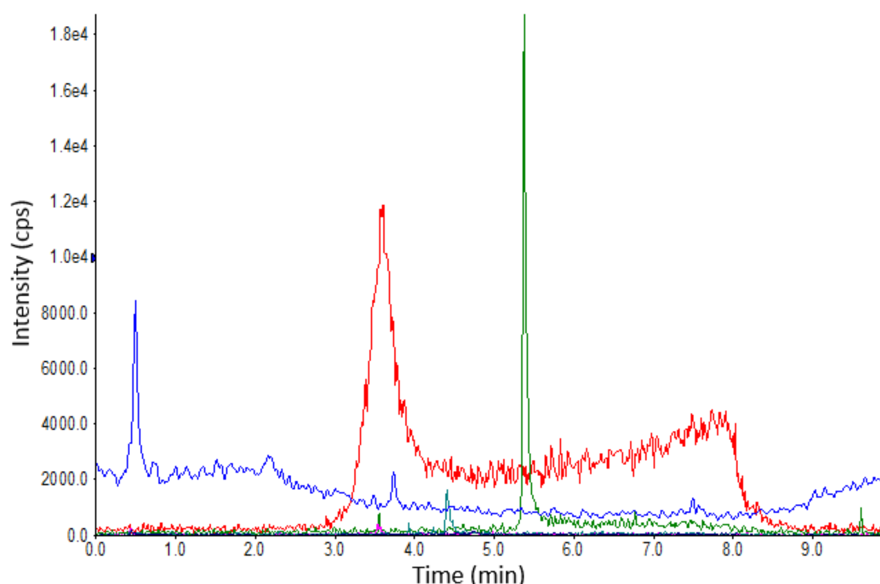

Figure 3.2 MRM chromatogram of an extracted drug free sample of authentic hair after screening for drugs of abuse and their metabolites. Peaks representing MP (red) and MQL (green) can be observed in the chromatogram.

3.3.2.5 Stability of quality control extract

After the analysis of the study samples for EtG, described in Chapter 2, the extracts from the hair samples were kept at ~ -80°C for approximately four months before being screened for drugs of abuse using the method described in this chapter. Therefore, the stability of the analytes under these conditions was investigated. The results are presented in Table 3.7.

The % CV for each of the analytes in both the fresh and stored QC extracts was less than 4%. For five of the six analytes the % difference between the average peak areas for the stored and fresh QC extracts was less than 15%, indicating that these analytes were stable in the extracts stored at ~ -80°C for four months. The % difference between the average peak areas for AP was 17.4%. This result was only slightly over the recommended guideline of a % difference of less than 15% to demonstrate stability [210] and in the context of this screening method was not considered to be a major concern.

Table 3.7 Stability of the drugs of abuse and metabolites in extracts stored at ~ -80°C for four months

Analyte	Fresh QC extract			Stored QC extract			% Difference
	Average peak area	STDEV	% CV	Average peak area	STDEV	% CV	
AP	344833	10534	3.1	404833	8232	2.0	**17.4**
MP	1665000	10488	0.6	1885000	31464	1.7	**13.2**
COC	9383333	169312	1.8	9315000	86891	0.9	**-0.7**
BZE	444667	4546	1.0	460500	3271	0.7	**3.6**
CE	932833	12937	1.4	940000	13711	1.5	**0.8**
MQL	1815000	27386	1.5	1950000	12649	0.6	**7.4**
n	6			6			

3.3.3 Analysis of study samples

The qualitative method described and validated in this chapter was used to screen the 257 study sample extracts for the presence of any of the following drugs of abuse and/or metabolites: AP, MP, COC, BZE, CE and MQL. Samples were considered to be positive for any of the drugs of abuse and/or metabolites listed above if the peak area for a specific analyte in the sample was greater than the average peak area for that analyte in the cutoff QCs analysed within the same batch.

For each of the sample batches the cutoff QC was prepared in duplicate and injected after every ten sample injections. The purpose of this was to monitor the stability of the LC-MS/MS system in the absence of any internal standard. The results presented in Table 3.8 indicate that the LC-MS/MS system was stable during the analysis of each of the five sample batches. The intra batch % CV for each of the analytes was less than 15%, except for the % CV of AP in sample batch 4 which was 15.4%.

Table 3.8 % CV of each of the six analytes in the cutoff QC injected repeatedly throughout each of the five sample batches to demonstrate stability of the LC-MS/MS system in the absence of an internal standard

	Sample Batch				
	1	2	3	4	5
Analyte	**% CV**				
AP	6.7	4.6	13.8	15.4	6.8
MP	10.8	9.2	12.6	13.6	8.0
COC	6.7	5.1	8.1	10.1	5.1
BZE	6.1	3.9	8.2	11.7	5.8
CE	5.8	4.0	6.9	9.6	5.1
MQL	10.8	6.5	9.3	10.9	6.1
n	12 of 12	12 of 12	12 of 12	12 of 12	8 of 8

After comparing the peak areas of any peaks detected in the samples with the respective peak areas in the cutoff QCs, only 5.4% (n = 14) of the 257 samples analysed were determined to be positive for any of the drugs of abuse and/or metabolites in the screening method. These positive hair samples were collected from 8 of the 135 study participants at various time points throughout the duration of the broader adherence study. More than half (62.5%) of the study participants identified to be chronic drug users were also previously determined to be heavy drinkers. The results are presented in Table 3.9.

Table 3.9 Drugs of abuse and their metabolites detected in positive hair samples collected from study participants at weeks 16, 32 and 48

Participant number	Drugs of abuse/metabolites detected			Heavy drinker
	week 16	week 32	week 48	
26	AP, MP, MQL	ns	MP, MQL	✓
32	MQL	MQL	MQL	×
76	ns*	COC, BZE, CE	ns	✓
82	MQL	ns	MQL	✓
84	ns	MQL	ns	×
85	AP, MP, MQL	ns	ns	×
101	AP, MP	AP, MP	AP, MP	✓
118	BZE	ns	ns	✓

* ns = no sample provided

It can be seen from the results presented in Table 3.9 that participants who provided more than one hair sample during the 48 week study period were consistent in their drug use. For example, participant 101 was positive for AP and MP at weeks 16, 32 and 48. Interestingly, 64% of the positive samples contained MQL.

In addition to MP and MQL, AP was also detected at week 48 for participant number 26, however the peak area of the AP peak was below the average peak area of the AP peaks in the cutoff QCs and therefore the sample could not be reported as positive for AP. Similarly, in addition to BZE, COC was detected at week 16 for participant number 118, however the peak area of the COC peak was below the average peak area of the COC peaks in the cutoff QCs and therefore the sample could not be reported as positive for COC.

Participant number 76 was positive for COC, BZE and CE at week 48. The BZE:COC ratio was 0.91, which is greater than the suggested ratio of 0.05 for confirming a positive COC result [172]. This participant, previously classified as a heavy drinker with a concentration of EtG approximately six times the concentration of the cutoff to identify heavy drinking, was also positive for the metabolite CE, indicating that alcohol and COC were used at the same time. Furthermore, the presence of the CE metabolite removes any concern that the COC positive result was due to external contamination.

Analyte peak areas in positive samples were at least double the average peak areas of the respective analytes in the cutoff QCs. Figure 3.3 shows the high intensity peaks that were observed for each of the six analytes extracted from positive authentic samples of hair.

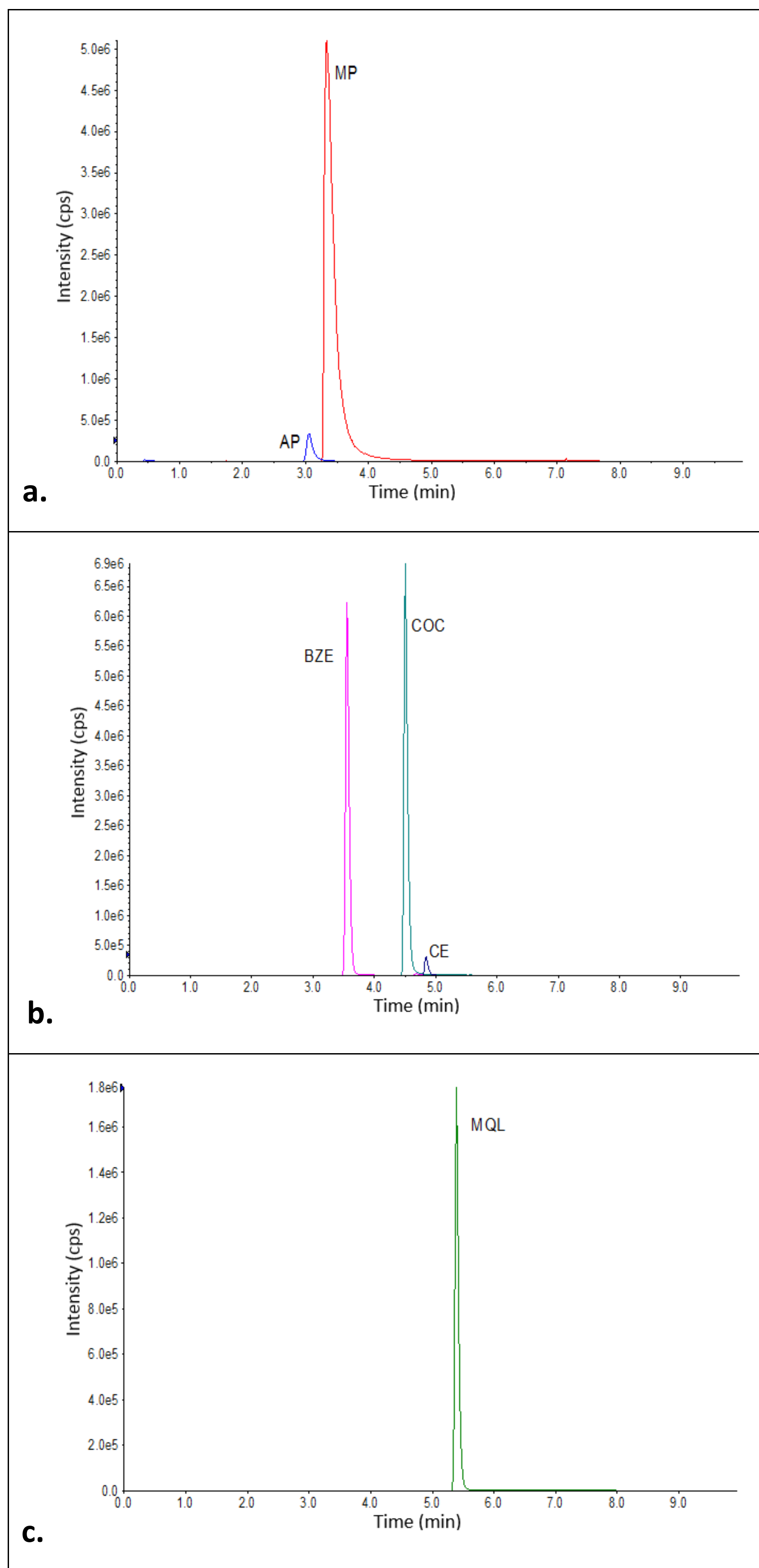

Figure 3.3 MRM chromatograms from three hair samples positive for **a.** AP and MP (participant 101); **b.** COC, BZE and CE (participant 76) and **c.** MQL (participant 32).

From the peak areas observed in the positive samples it can be assumed that the concentrations of drugs present in the hair samples well exceeded the SoHT recommended cutoff concentrations to identify chronic drug abuse [56]. If compared with the intensity of the peaks observed in the chromatogram of the cutoff QC (Figure 3.1), the intensity of the peaks represented in Figure 3.3 is much greater. Furthermore, for each of the positive hair samples, except for the samples collected from participant 26 at week 48 and participant 118 at week 16, the corresponding metabolite for the parent compound was also detected at levels above recommended cutoff levels. Therefore, there is little uncertainty that any false positive results were reported.

However, it is likely that false negative results have been reported. One of the limitations of the sample analysis process was that there was not enough hair available to extract samples using a method specifically developed to extract drugs of abuse from hair. Whilst various extraction procedures have been reported in literature, extraction with methanol is generally considered to be compatible with all drugs of abuse [62]. Methanol, an organic solvent, is able to dissolve both neutral and lipophilic compounds. However, since the samples in this context were originally extracted for the quantification of EtG, a polar molecule, water was used as the extraction solvent. These water extracts were later analysed for drugs of abuse using the developed screening method. Therefore, as a result of poorer solubility in water, it is possible that only a small percentage of drugs present in the hair samples were extracted. Unfortunately, it was not possible to re-extract the samples of hair using methanol after the water extraction. This might have given a better indication of the drugs of abuse present in the samples.

Despite this limitation, it is evident from the results that drugs of abuse and their metabolites were extracted from some hair samples and could be detected. In addition to the positive results reported above there were many samples with easily detectable analyte peaks with large peak areas. However, after comparison with the respective peak areas in the cutoff QC, the levels were below the recommended cutoff values and therefore not reported as positive. This was often the case when MQL was detected in

samples and suggests that the cutoff chosen was possibly too high, resulting in the reporting of false negatives.

Whilst the cutoffs recommended by the SoHT are to identify chronic drug use, drug concentrations in the low pg/mg range have been detected after once-off drug use [56], complicating the interpretation of results. Therefore the analytes detected in some of the samples, but with peak areas below the cutoff levels, could have been as a result of once-off or infrequent drug use. However, there are too many uncertainties to further investigate this possibility in the context of this study.

3.4 Summary and Conclusions

Described in this chapter is the validation of a screening method to detect the presence of some of the commonly used drugs of abuse in South Africa in samples of hair. In the absence of defined international guidelines for the validation of qualitative methods, recommendations from previously validated qualitative methods [228–230] were applied to validate this screening method. The cutoff concentrations recommended by the SoHT to identify chronic drug use [56] were used to prepare a single QC at the respective cutoff concentrations for each of the drugs and metabolites included in the drug screen. The precision of the assay was demonstrated by the extraction and analysis of the cutoff QC over three validation batches. Even in the absence of an internal standard, the intraday and interday % CV for each of the six analytes in the cutoff QC fell within acceptable limits.

The effect of matrix components on the ionisation of each of the analytes was investigated, and in the case of CE the variation between the peak areas in the ten different matrices was greater than 30%. This result suggests that the ionisation of CE is likely affected by components within the hair matrix, and in the absence of an internal standard, these effects were not compensated for. Stability experiments with stored QC

extracts indicated that the storage of extracts at ~ -80°C for four months before screening did not significantly affect the stability of extracted drugs.

The stored extracts from the study samples were analysed for AP, MP, COC, BZE, CE and MQL using the validated screening method and the prevalence of chronic drug use in this study population was determined to be 5.9%. The results show that drug use was consistent among individuals over the 48 weeks, and that MQL was the most commonly detected drug of abuse. Due to the limitations and uncertainties surrounding the extraction of the drugs of abuse and metabolites, it is likely that false negative results were reported. As a result the actual prevalence of chronic drug use could be higher than 5.9%.

However, despite the limitations that have been discussed, the results presented in this chapter provide insight into patterns of drug use among HIV-infected patients in Cape Town, South Africa. Furthermore, screening for drugs of abuse using hair as a matrix is new to South Africa and this screening method forms a basis for developing more sensitive methods to detect commonly abused drugs as well as drugs unique within the South African context. As previously mentioned, there are many different practical applications of hair analysis and, therefore, there is considerable potential for this method of drug analysis within the country as a complementary matrix to blood and urine.

4 Quantitative determination of efavirenz in hair

4.1 Introduction

EFV is a NNRTI used for the treatment of HIV infection worldwide [7] and is an important component of HAART. EFV has a long half-life of 52 to 76 hours following a single oral dose, and 40 to 55 hours after multiple doses. As a result of its long half-life EFV is administered once a day as a single 600 mg dose [5]. In South Africa, the most effective ART combination includes EFV in combination with tenofovir and either emtricitabine or lamivudine [4].

TDM plays an important role in monitoring a patient’s response to ART by measuring ARV drug levels. Sub-therapeutic levels of EFV can put patients at risk for treatment failure as well as for the development of drug resistance, whereas super-therapeutic EFV levels have been associated with CNS side effects [8]. It is therefore important for successful response to ART to ensure that drug levels are maintained within therapeutic ranges.

One of the most common reasons for sub-therapeutic levels of ARV drugs is poor adherence [231]. Current measures used to measure adherence include patient self-report, pill counts, prescription refills and electronic medication monitors [21]. However, each of these methods comes with certain limitations. Self-report measures, for example, often overestimate adherence [18].

A more direct method of measuring adherence is by measuring drug exposure as is performed in TDM. Conventionally, drug exposure is measured by determining concentrations of a drug, or its metabolite, in plasma [21]. However, plasma concentrations usually only provide a short-term (hours to 1 to 2 days) assessment of drug exposure and therefore cannot predict long-term treatment outcomes [27]. Additionally, high intra-individual variability in concentrations of ARV drugs may limit the ability to use a single plasma measurement in TDM [25]. Plasma drug concentrations are also susceptible to ‘white coat effects’ where patient adherence improves just prior

to clinic visits [26]. Therefore, a single drug concentration in plasma might not reflect an accurate measure of actual drug exposure [198].

Plasma EFV concentrations are also characterised by high inter-individual variability in concentrations which are explained, in part, by polymorphisms in the CYP2B6 gene [9]. This gene encodes the cytrochome P450 enzyme CYP2B6 which is mainly responsible for the metabolism of EFV [10], and CYP2B6 genetic polymorphisms have been shown to influence the metabolism of EFV and therefore plasma levels [9]. Additionally, research has shown that the prevalence of genetic slow metabolisers is significantly higher in sub-Saharan African populations [232].

More recently, hair has been studied as an alternative matrix for TDM. Drug levels in hair provide a longer window of detection (weeks to months) and therefore hair analysis has the advantage of assessing long-term adherence [27] and provides an estimation of the average level of drug exposure over time [233]. Concentrations of ARVs in hair have been shown to be closely correlated with virologic outcomes [185–193]. Bernard et al. [188], first showed in 1998 that levels of the PI, indinavir, in hair samples were significantly higher in HIV-infected patients who responded to HAART compared to patients who did not respond to HAART. Moreover, concentrations of EFV in hair have also been shown to be strongly correlated with virologic outcomes [190]. Additionally, recent studies have demonstrated that concentrations of EFV in hair are also influenced by CYP2B6 polymorphisms [202, 233].

As a result of the potential that hair analysis holds for TDM and adherence studies, LC-MS/MS methods have, in recent years, been developed for the analysis of ARVs in hair. Studies have reported sensitive LC-MS/MS methods for the analysis of the ARVs, EFV, lopinavir and ritonavir [197], nevirapine [198], as well as abacavir and tenofovir [199] in hair. To date the abovementioned method, developed by Huang et al. [197], is the only validated method that has been published for the analysis of EFV in hair. This method was validated over the range of 0.05 – 20 ng/mg using 2 mg of hair. EFV was extracted from cut hair samples along with two other ARVs, lopinavir and ritonavir, during an

overnight incubation step. The validated method was used to analyse samples of hair collected from patients on EFV-based HAART. The results of the hair analysis showed that the median EFV concentration in the hair of patients responding to treatment (3.4 ng/mg) was significantly higher than the median EFV concentration in the hair of patients not responding to treatment (0.68 ng/mg) [197].

Whilst the EFV method discussed above has been used for the determination of EFV levels in hair in the context of other studies [191, 202, 233, 234], the amount of published data regarding EFV levels in hair is limited, especially in the South African context where EFV is widely prescribed to HIV-infected patients. There is only one study that reports EFV levels in hair samples collected from South Africans [202]. In this study hair samples were collected from HIV-infected South African females and sent to an international laboratory where the method developed by Huang et al. [197] was used to analyse the samples. The median EFV concentration in the hair samples was reported to be 5.60 ng/mg. EFV levels in hair were not found to be associated with virologic response [202].

Therefore, there is a need for the development of validated methods for the analysis of ARVs in hair in South Africa to compliment current methods of TDM and to provide objective measures of assessing long-term adherence. The main aim of this chapter is to describe the development and validation of such a method. The LC-MS/MS method for the quantitative determination of EFV in hair described in this chapter is the first such method to be developed in South Africa, and the results from the sample analysis provide insight into the possibility of implementing the use of hair as a matrix for monitoring ARV drug exposure in South Africa.

4.2 Materials and Methods

4.2.1 Collection and storage of hair samples

4.2.1.1 Blank and EFV positive samples

EFV free hair, collected from a healthy child, was used during method development, preparation of calibration standards and QCs and validation experiments. Seven additional sources of EFV free hair for matrix effects and specificity experiments were collected from healthy adult volunteers. Hair positive for EFV was collected from an adherent patient on an EFV-based ART regimen and was used during method development experiments and throughout the study as an authentic QC.

A hair repository was registered with the University of Cape Town, Faculty of Health Sciences, Human Research Ethics Committee for the collection and storage of hair samples (reference R026/2015). Volunteers provided verbal consent to their hair being collected. All hair samples registered in the repository were stored in paper envelopes at room temperature.

4.2.1.2 Study samples

Chapter 2, Section 2.2.1.2, provides details regarding the hair samples collected for the broader adherence study. Due to the limited amount of hair that was collected from the HIV-infected patients, the method in this chapter was developed to require only 0.2 mg of hair.

4.2.2 Chemicals and reagents

EFV and its deuterated analogue (EFV-d5), used as an internal standard, were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Stock solutions of 1 mg/ml were prepared in methanol (for EFV) and acetonitrile (for EFV-d5). Working solutions were prepared by diluting EFV and the internal standard to 10 and 1 µg/ml with methanol. All stock and working solutions were stored at ~ -80°C. Methanol (gradient

grade) was purchased from Merck (Darmstadt, Germany) and acetonitrile (LC-MS grade) was purchased from Honeywell International Inc. (Mexico City, Mexico). Formic acid (~ 98%) was purchased from Fluka Analytical (Buchs, Switzerland). Deionized water was prepared using a Synergy Water Purification System purchased from Merck Millipore (Massachusetts, USA).

4.2.3 Extraction method

For the extraction of EFV, 0.2 mg of hair was weighed into 2 ml screw-cap tubes to which two metal balls (2.4 mm) were added. The metal balls and screw-cap tubes were purchased from Omni International Inc. (Georgia, USA). Extraction solvent (250 µl) was added to each tube before pulverizing the hair samples. The extraction solvent was made up of a mixture of methanol and water (70:30, v/v) with the internal standard added at a concentration of 10 ng/ml. Samples were vortex mixed briefly for 10 s and pulverized using the Omni Bead Ruptor 24 (Omni International Inc., Georgia, USA) at a speed of 5 $m.s^{-1}$ for five cycles of 60 s each, with a dwell time of 30 s after each cycle. After the samples were pulverized, the extract was removed and filtered through a 0.45 µm filter under vacuum (Merck Millipore, Massachusetts, USA). The clear extract (10 µl) was injected into the LC-MS/MS system for analysis.

During the method development stages the simultaneous pulverization and extraction method described above was compared to two other methods in order to determine which method was the most effective at removing EFV from samples of EFV positive authentic hair. The other two methods had a slightly longer incubation time with either a 10 or 30 min ultrasonication step. Authentic hair (0.2 mg) was weighed into screw-cap tubes for each of the three extraction methods being investigated. Each experiment was carried out in triplicate. One set of the weighed authentic hair samples was extracted according to the method described above. Two metal balls were added to the other two sets of samples which were then pulverized at 5 $m.s^{-1}$, as described above. After the pulverization step, 250 µl extraction solvent was added to each sample, following which the samples were vortex mixed briefly for 10 s and ultrasonicated for either 10 or 30

min. After the required ultrasonication time the extracts were removed and filtered as described above. The extracts from each of the three methods were analysed to compare the amount of EFV extracted in each of the experiments.

In deciding on the extraction solvent to be used, the amount of EFV extracted with 100% methanol was compared to the amount extracted with only 70% methanol. Authentic hair, 0.2 mg, was extracted according to the simultaneous pulverization and extraction method described above with either 100% methanol or 70% methanol used as the extraction solvent. Each experiment was repeated six times. The extracts from each of the experiments were analysed to determine any differences in the amount of EFV extracted for each of the experiments.

4.2.4 LC-MS/MS Conditions

EFV was analysed using an Agilent Technologies 1260 Infinity Liquid Chromatography system (California, USA) coupled to an AB SCIEX Q Trap 5500 mass spectrometer (SCIEX, Massachusetts, USA). Chromatographic separation was achieved using an Agilent Poroshell 120 C18 column (2.7 µm, 4.6 x 50 mm) (Agilent Technologies, California, USA) kept at 30°C. The system was run using an isocratic elution with mobile phase made up of 0.1% formic acid in water and acetonitrile (20:80, v/v). The total run time was 3 min at a constant flow of 500 µl/min.

The best sensitivity for EFV was achieved when the mass spectrometer was set to electrospray ionization in positive MRM mode. The mass spectra for EFV (Figure 4.1) and EFV-d5 (Figure 4.2) were obtained by direct infusion of each of the reference standards into the mass spectrometer. The reference standards were diluted to 100 ng/ml in 0.1% formic acid in water and 0.1% formic acid in acetonitrile (50:50, v/v). EFV was monitored using two transitions, 316 → 244 (quantifier ion) and 316 → 232 (qualifier ion). EFV-d5 was monitored at 321 → 246.

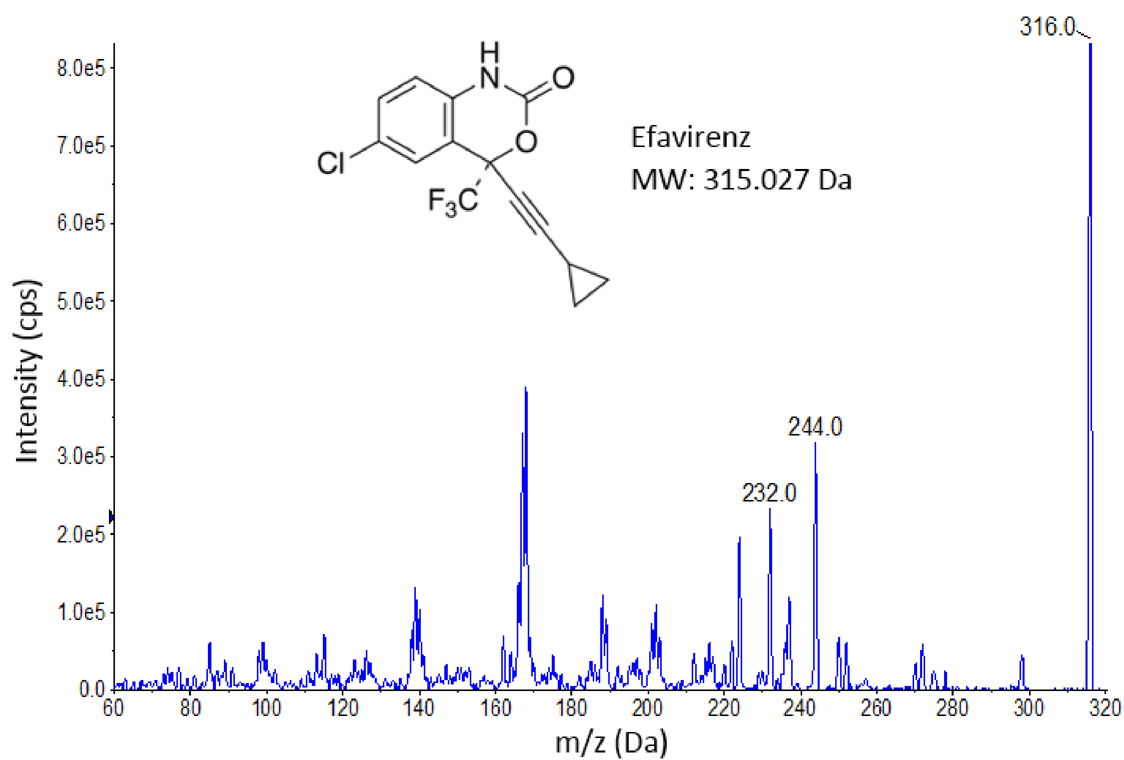

Figure 4.1 Initial product scan mass spectrum of efavirenz.

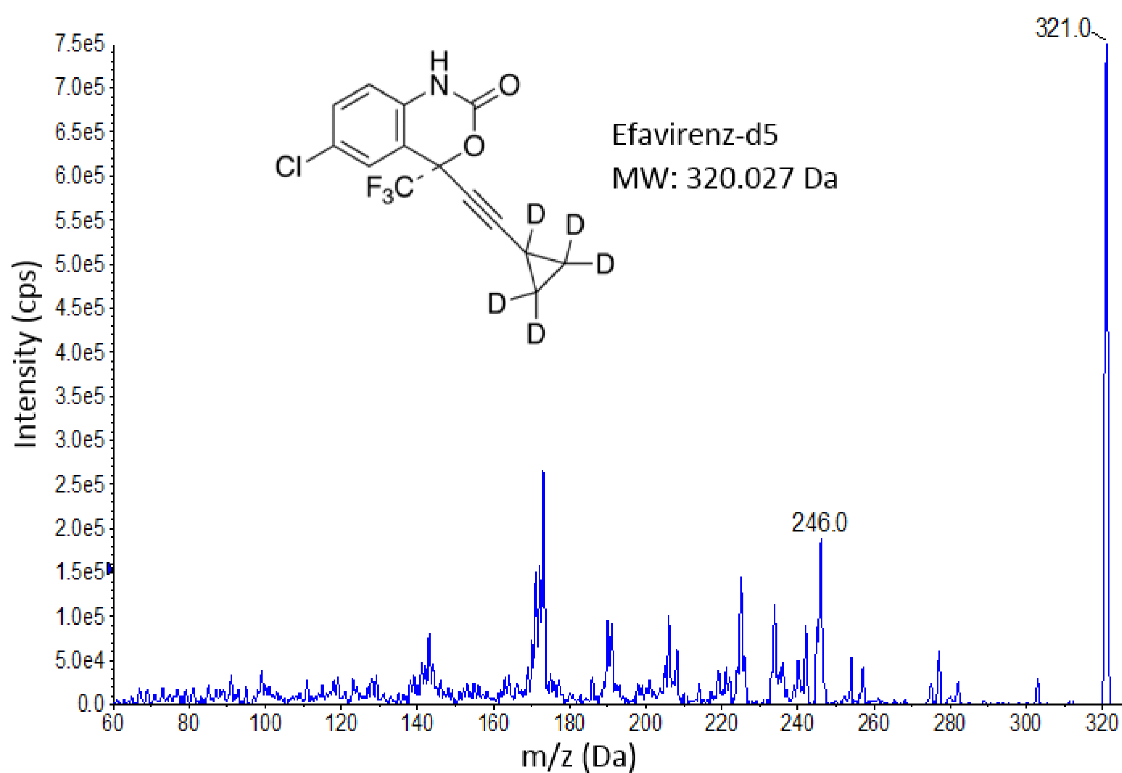

Figure 4.2 Initial product ion mass spectrum of efavirenz-d5.

Flow injection analysis was used to optimize the mass spectrometer conditions for the analysis of EFV. The ion spray voltage was set at 5500 V with a source temperature of 500°C. The nebulization, heating and curtain gases were set to 60, 60 and 30 psi, respectively. The collision gas was nitrogen with the pressure set to medium. The MRM transitions and final mass spectrometer conditions are described in Table 4.1. Analyst software version 1.6.2 (SCIEX, Massachusetts, USA) was used to analyse the data collected.

Table 4.1 MRM transitions and final mass spectrometer conditions for the analysis of efavirenz and efavirenz-d5

Analyte	Transition	Dwell Time (ms)	Declustering Potential (V)	Entrance Potential (V)	Collision Energy (V)	Cell Exit Potential (V)
EFV	316 → 244	200	81	10	19	20
	316 → 232	200	81	10	21	18
EFV-d5	321 → 246	200	86	10	17	24

4.2.5 Method validation

4.2.5.1 Preparation of calibration standards and QCs

EFV free hair was used for the preparation of the calibration standards and QCs. The preparation of the calibration standards and QCs was based on the extraction method developed for authentic hair samples as described above, although, the order of the steps were altered slightly to reduce the time taken to prepare the spiked standards and QCs. Additionally, blank hair was extracted in larger quantities to simplify the process; 0.8 mg rather than 0.2 mg of hair was extracted in each screw-cap tube. Extraction solvent (1 ml) with 10 ng/ml (12.5 ng/mg) internal standard was added to 0.8 mg blank hair in screw-cap tubes containing two metal balls. The hair samples with extraction solvent were vortex mixed briefly for 10 s and pulverized according to the method

previously described. The extracted solvent from each tube was removed and pooled together in a separate larger tube.

The extracted solvent was used to prepare the calibration standards and QCs. For the calibration standards EFV was spiked into extracted solvent at a concentration of 32 ng/ml and serially diluted 1:1 with extracted solvent to produce concentrations of 16, 8.0, 4.0, 2.0, 1.0 and 0.5 ng/ml (corresponding to the standards 40, 20, 10, 5.0, 2.5, 1.25 and 0.625 ng/mg).

For the preparation of the high, medium and low QCs as well as the LLOQ, EFV was spiked into extracted solvent at a concentration of 26 ng/ml (high QC) and diluted with extracted solvent to produce concentrations of 13 ng/ml (medium QC), 1.25 ng/ml (low QC) and 0.5 ng/ml (LLOQ) (corresponding to high, medium, low QCs and the LLOQ at 32.5, 16.25, 1.56 and 0.625 ng/mg respectively). The calibration standards and QCs were kept at ~ -80°C until required. For each of the three validation batches, the calibration standards were analysed in duplicate and the QCs in six-fold. Before injection into the LC-MS/MS system, the standard and QC solutions were filtered as previously described. Calibration curves were constructed by plotting peak area ratio (peak area of the analyte peak/peak area of the internal standard peak) against analyte concentration.

Aliquots from a homogenous sample of authentic hair, positive for EFV, were weighed into screw-cap tubes (0.2 mg). These samples of hair were extracted according to the extraction method previously described in Section 4.2.3 and analysed as authentic QCs. Authentic QCs were included for analysis in each of the validation and sample batches to monitor the reproducibility of the extraction procedure.

4.2.5.2 Validation experiments

The method was validated according to the international guidelines of the Food and Drug Administration (FDA) [208, 209] as well as the European Medicines Evaluation Agency (EMEA) [210, 211]. The following validation experiments were performed: stock

solution accuracy and stability, accuracy and precision, matrix effects, specificity, sensitivity, carry-over and autosampler stability.

Stock solution accuracy and stability

Measurements for the comparison of solutions for the determination of stock solution accuracy and stability were made using an Agilent 8453 ultraviolet (UV) - visible Spectroscopy System (Agilent Technologies, California, USA). Measurements were taken at two fixed wavelengths of 204 and 247 nm. A mixture of methanol and water (70:30, v/v) was used as a system blank. To determine stock solution accuracy, two independent stock solutions of EFV (SS1 and SS2) were prepared by two different analysts on the same day at 1 mg/ml in methanol. The stock solutions were diluted, in triplicate, to 10 µg/ml with a mixture of methanol and water (70:30, v/v). The absorbance of each dilution was measured at two fixed wavelengths (λ = 204 nm and λ = 247 nm) and compared.

To determine short-term stock solution stability, 1 mg/ml aliquots of SS2 were stored at room temperature (~ 22°C), ~ 4°C and ~ -20°C (test solutions) for 72 hours. The absorbance of each test solution and the reference solution (kept at ~ -80°C), prepared in triplicate at 10 µg/ml with a mixture of methanol and water (70:30, v/v), was measured at two fixed wavelengths (λ = 204 nm and λ = 247 nm) and compared. Long-term stock solution stability (61 days at ~ -80°C) was determined by comparing SS2 to a reference stock solution prepared on the day of stability testing (SS3) using UV-visible spectrophotometry as described above.

4.2.6 Extraction efficiency

To investigate the efficiency of the extraction method, authentic hair was weighed (0.2 mg) into 2 ml screw-cap tubes and extracted, in six-fold, according to the extraction method described above. The extract was removed for analysis and the hair was left to air dry at room temperature. This extraction process was repeated on the authentic hair two more times. The extracts from each of the extractions were filtered and analysed

using the LC-MS/MS method previously described in order to compare the amount of EFV that remained in the hair after each of the extractions.

4.2.7 Analysis of study samples

The 257 study samples were quantitatively analysed using the validated method described above. Small samples of hair (∼ 0.2 mg) were cut from the proximal section of each of the collected hair samples (about 1 cm) with scissors, placed in 2 ml screw-cap tubes and weighed. The mass of the tube was subtracted from the total mass in order to get the exact mass of the hair sample, which was recorded. The average mass of hair weighed was 0.28 mg (±0.047). The hair samples were processed as described in Section 4.2.3 above.

4.2.8 Statistical analysis

GraphPad Prism 4 (California, USA) was used for the statistical analysis of data. Statistical significance was tested using ANOVA (95% CI).

4.3 Results and Discussion

4.3.1 Method development and optimization

4.3.1.1 Extraction method

A simple and quick method was developed and optimized for the extraction of EFV from small samples of hair, without the inclusion of a decontamination/wash step. As EFV is taken orally external contamination of hair by EFV was not considered to be a concern and therefore a wash step was considered unnecessary. An earlier study for the analysis of ARVs in hair has also discussed the irrelevance of a wash step when analysing hair for ARV drugs [198].

The extraction method developed was based on pulverizing samples of hair into a fine powder and in order to optimize the extraction process, three different methods were tested using authentic hair. The amount of EFV removed from the authentic hair during each of the extraction methods was compared (Figure 4.3). Statistical analysis (ANOVA, 95% CI) of the quantity of EFV extracted indicated no statistical difference between the means of the EFV peak areas for each of the three methods investigated. It was therefore decided to develop the extraction method using the simultaneous pulverization and extraction procedure as out of the three methods investigated this was the easiest and most time efficient.

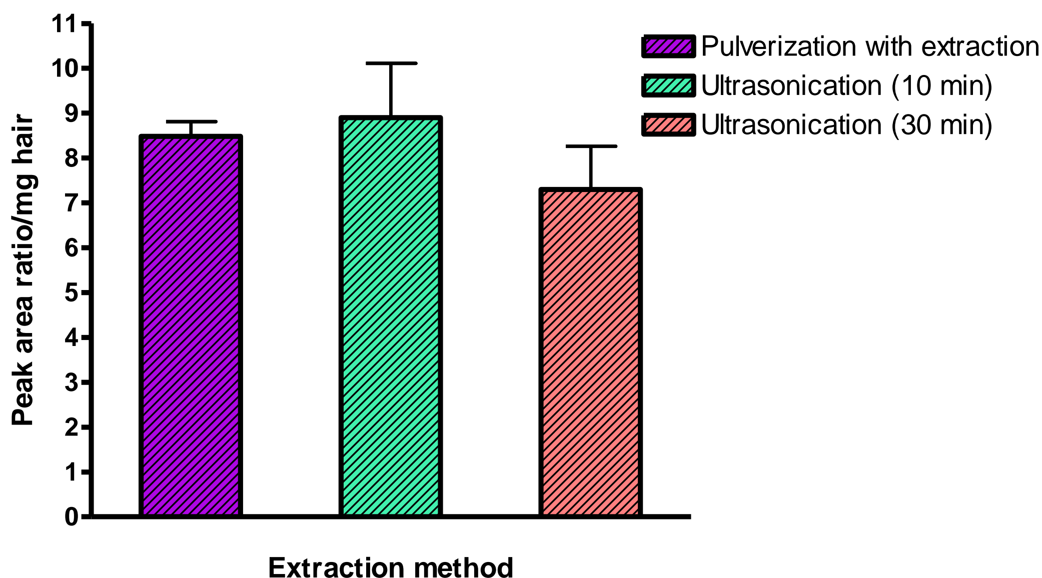

Figure 4.3 Comparison of the amount efavirenz extracted from authentic hair after extraction with three different methods. Methods included either a simultaneous pulverization and extraction step, a 10 minute or a 30 minute ultrasonication step. Error bars show the standard deviation from the mean value (n = 3).

As discussed in Chapter 2, pulverizing samples of hair prior to extraction has been shown to increase extraction efficiency and decrease the required extraction time for certain drugs. However, pulverizing samples of hair when analysing it for ARVs seems to not influence the amount of drug extracted and a comparable amount of drug is extracted

when hair samples are cut into small segments [197, 198]. In the development of methods for the analysis of ARVs in hair, reported by Huang et al. [197, 198], cutting samples of hair was preferred over pulverizing or grinding the hair into a fine powder. The main reason for this being that the type of grinding procedure used involved freezing samples of hair with liquid nitrogen before grinding - a complicated and time consuming step. The pulverization process used in this study, however, was simple and quick. Additionally, it was found that when working with very small amounts of hair pulverizing hair samples was easier than cutting hair into small segments.

Whilst methanol has been previously used for the extraction of ARVs from hair [197], the extraction solvent used in this method consisted of a 70:30 (v/v) mixture of methanol and water. The reason for this was to improve the chromatography by making the extraction solvent, and therefore the injection solvent, more compatible with the chromatography conditions. The effect of adding water to the methanol extraction solvent on the amount of EFV extracted from authentic hair was investigated to determine whether this resulted in a decrease in the extraction efficiency. Results from this experiment indicated that only approximately 5% less EFV was extracted when using 70% methanol compared to 100% methanol as the extraction solvent.

Due to the limited amount of hair available for the analysis of the study samples, this method was developed using small amounts of hair. EFV is a lipophilic drug that accumulates well in hair due to its basic properties [200], and therefore high levels of EFV are found in hair making it possible to detect in even small amounts of hair. In previously published methods ARVs, including EFV, have been extracted from 2 mg of hair [197, 198]. In one study the ARV, nevirapine, was extracted from a single strand of hair weighing approximately 0.1 mg [198]. In the current method 0.2 mg of hair was used. As shown in Figure 4.4, the EFV peak was easily detected after the extraction of EFV from 0.2 mg of authentic hair.

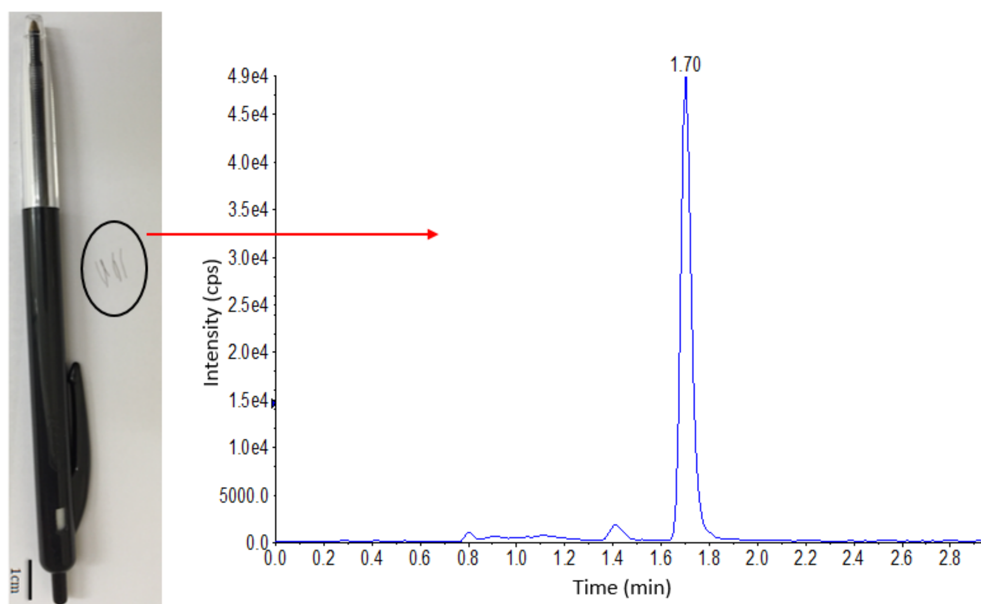

Figure 4.4 The chromatogram on the right shows the easily detectable efavirenz peak after extraction and LC-MS/MS analysis of 0.2 mg authentic hair, shown encircled on the left (pen shown for size comparison).

4.3.1.2 Chromatography

Two different columns were tested for the separation of EFV; the Agilent Poroshell column (2.7 µm, 4.6 x 50 mm) and the Waters Atlantis T3 column (3 µm, 2.1 x 100 mm) (Massachusetts, USA). Better peak shape was observed using the Poroshell column. Additionally, the retention time of EFV was less when using the Poroshell column compared to the Atlantis T3 column (approximately 1.7 min compared to approximately 2.5 min), allowing for a short run time of only 3 min. A representative chromatogram of the medium QC is shown in Figure 4.5.

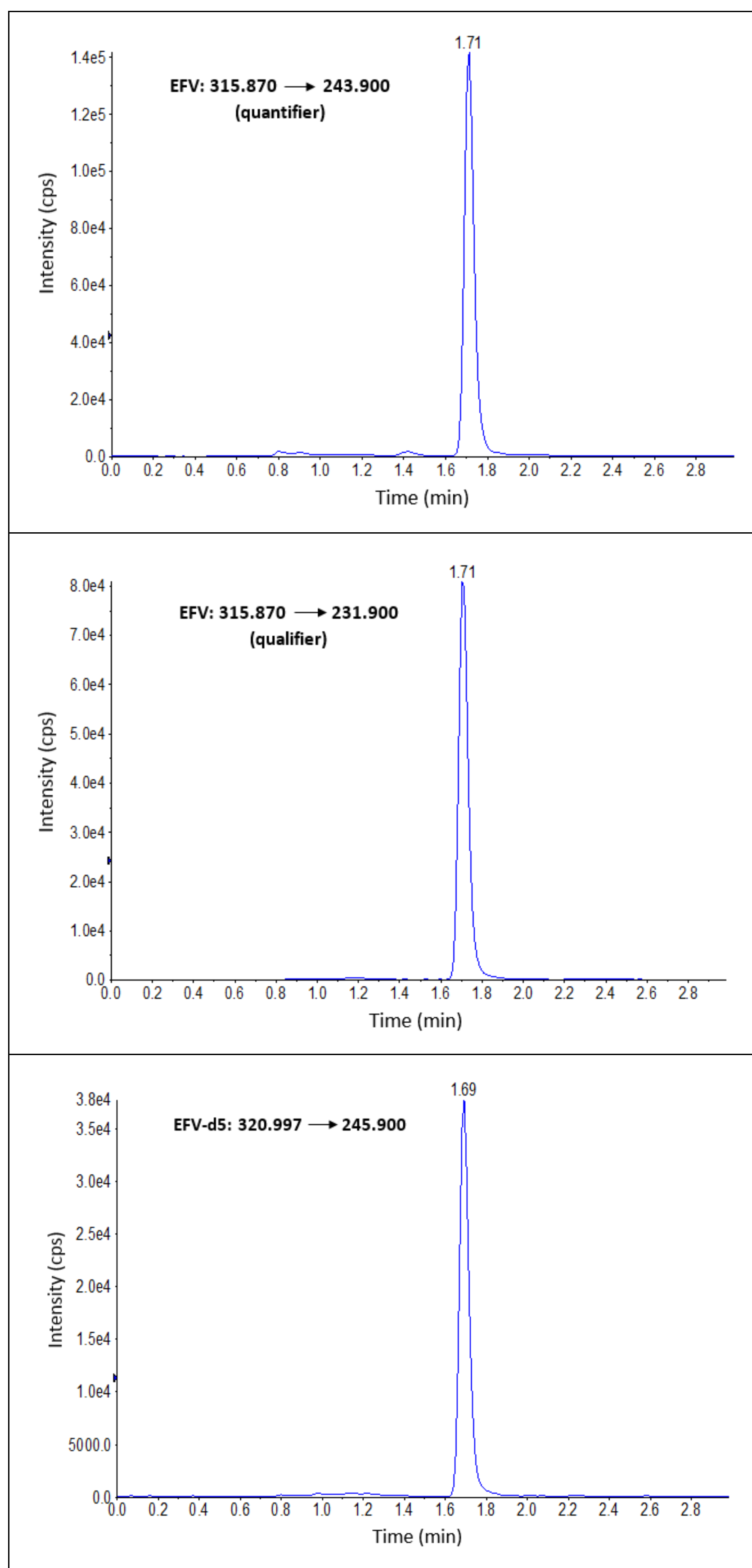

Figure 4.5 MRM chromatograms of blank hair spiked with efavirenz (16.25 ng/mg) and internal standard (12.5 ng/mg).

4.3.2 Method validation

4.3.2.1 Stock solution accuracy and stability

Accuracy was determined by comparing two independent stock solutions (SS1 and SS2) of EFV using UV-visible spectrophotometry. The results are shown in Table 4.2.

Table 4.2 Stock solution accuracy of efavirenz stock solutions 1 and 2

	Absorbance at λ = 204 nm (AU)		Absorbance at λ = 247 nm (AU)	
	SS1	**SS2**	**SS1**	**SS2**
Absorbance A	1.15	1.12	0.527	0.506
Absorbance B	1.16	1.12	0.530	0.506
Absorbance C	1.15	1.12	0.527	0.506
Average	1.15	1.12	0.528	0.506
STDEV	0.00603	0.000643	0.00140	0.000434
% CV	0.5	0.1	0.3	0.1
% Difference		**-2.5**		**-4.2**

For the stock solutions to be accurate, the % difference between the two stock solutions should not be more than 5%. Therefore, the two stock solutions prepared were shown to be accurate, with a % difference in absorbance of -2.5% and -4.2% at λ = 204 nm and λ = 247 nm respectively.

Short-term stock solution stability was demonstrated for 72 hours at room temperature (~ 22°C), ~ 4°C and ~ -20°C. The % difference between the absorbance of the reference and test solutions kept at each of the described conditions was less than 4% at λ = 204 nm (Table 4.3) and less than 7% at λ = 247 nm (Table 4.4).

Table 4.3 Short-term stock solution stability of efavirenz kept at room temperature (~ 22°C), ~ 4°C and ~ -20°C for 72 hours. Absorbance readings measured at the wavelength 204 nm are presented

	Absorbance at λ = 204 nm (AU)			
	Reference ~ -80°C	**Test ~ -20°C**	**Test ~ 4°C**	**Test room temp.**
Absorbance A	1.14	1.17	1.17	1.14
Absorbance B	1.13	1.17	1.17	1.13
Absorbance C	1.13	1.17	1.17	1.13
Average	1.13	1.17	1.17	1.13
STDEV	0.00252	0.00205	0.00142	0.00204
% CV	0.2	0.2	0.1	0.2
% Difference		**3.3**	**3.3**	**0.1**

Table 4.4 Short-term stock solution stability of efavirenz kept at room temperature (~ 22°C), ~ 4°C and ~ -20°C for 72 hours. Absorbance readings measured at the wavelength 247 nm are presented

	Absorbance at λ = 247 nm (AU)			
	Reference ~ -80°C	**Test ~ -20°C**	**Test ~ 4°C**	**Test room temp.**
Absorbance A	0.484	0.517	0.500	0.484
Absorbance B	0.483	0.515	0.500	0.482
Absorbance C	0.484	0.515	0.500	0.483
Average	0.484	0.516	0.500	0.483
STDEV	0.000698	0.000843	0.000343	0.000692
% CV	0.1	0.2	0.1	0.1
% Difference		**6.6**	**3.3**	**-0.2**

The absorbance readings of SS2 (prepared on the 29 Sep 2016) and SS3 (prepared on the 29 Nov 2016) to determine long-term stock solution stability of EFV are shown in Table 4.5.

Table 4.5 Long-term stock solution stability of efavirenz at ~ -80°C for 61 days

	Absorbance at λ = 204 nm (AU)		Absorbance at λ = 247 nm (AU)	
	SS2_29Sep2016	**SS3_29Nov2016**	**SS2_29Sep2016**	**SS3_29Nov2016**
Absorbance A	0.960	1.04	0.415	0.434
Absorbance B	0.958	1.04	0.414	0.432
Absorbance C	0.957	1.04	0.413	0.432
Average	0.958	1.04	0.414	0.432
STDEV	0.00161	0.00182	0.000975	0.000991
% CV	0.2	0.2	0.2	0.2
% Difference		**8.7**		**4.4**

The % difference of 8.7% at λ = 204 nm and 4.4% at λ = 247 nm is less than the recommended 15% [210] and therefore indicates that the EFV stock solutions prepared in methanol are stable at ~ -80°C for at least 61 days.

4.3.2.2 Calibration range

The method was validated over a calibration range of 0.625 – 40 ng/mg. Calibration curves were generated for each of the three validation batches that were run. A quadratic regression equation was used and weighted by 1/(concentration x concentration). Each of the calibration curves showed a good fit, with all R values above 0.99. A representative calibration curve is shown in Figure 4.6.

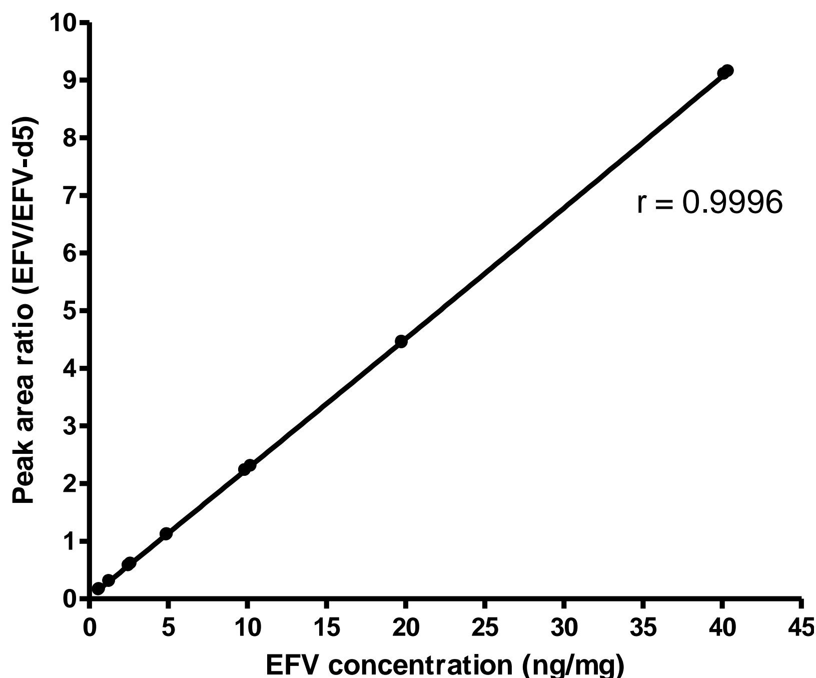

Figure 4.6 A representative calibration curve for efavirenz (Validation 2).

4.3.2.3 Accuracy and precision

The overall accuracy and precision of the validation process is determined by calculating the accuracy and precision statistics over the intraday and interday validation batches. Accuracy is expressed as the concentration of the analyte found as a percentage of the nominal concentration (% Accuracy) and precision is expressed as the coefficient of variation (% CV). For a valid method the intraday and interday accuracy is required to be within 15% over the entire calibration range and within 20% of the nominal concentration at the LLOQ. Additionally, the intraday and interday precision is required to be less than 15% over the entire calibration range and less than 20% at the LLOQ [208, 210]. The intraday and interday precision and accuracy results for the calibration standards are shown in Tables 4.6 and 4.7 respectively.

Table 4.6 Intraday accuracy and precision for efavirenz calibration standards

	Intraday						
	STD 1	**STD 2**	**STD 3**	**STD 4**	**STD 5**	**STD 6**	**STD 7**
Nominal concentration (ng/mg)	40.0	20.0	10.0	5.00	2.50	1.25	0.625
Average measured concentration (ng/mg)	40.1	19.8	10.2	4.89	2.49	1.27	0.621
n	2	2	2	2	2	2	2
STDEV	0.225	0.229	0.0301	0.125	0.0512	0.00513	0.0483
% CV	0.6	1.2	0.3	2.6	2.1	0.4	7.8
% Accuracy	100.2	98.9	102.2	97.8	99.7	101.8	99.3

Table 4.7 Interday accuracy and precision for efavirenz calibration standards

	Interday						
	STD 1	**STD 2**	**STD 3**	**STD 4**	**STD 5**	**STD 6**	**STD 7**
Nominal concentration (ng/mg)	40.0	20.0	10.0	5.00	2.50	1.25	0.625
Average measured concentration (ng/mg)	40.2	19.8	10.1	4.9	2.49	1.29	0.618
n	6	6	6	6	6	6	6
STDEV	0.234	0.146	0.155	0.0897	0.0989	0.0796	0.0286
% CV	0.6	0.7	1.5	1.8	4.0	6.2	4.6
% Accuracy	100.5	98.9	101.0	98.4	99.6	102.8	98.9

Both the intraday and interday accuracy and precision for the calibration standards were found to be within acceptable limits. The intraday % CV was below 8% and the % Accuracy between 97.8% and 102.2%. The interday % CV was below 7% and the % Accuracy between 98.4% and 102.8%.

The intraday and interday accuracy and precision results for the QCs, shown in Table 4.8, were also found to be within acceptable limits.

Table 4.8 Intraday and interday accuracy and precision for efavirenz quality controls

	Intraday				Interday			
	QC High	QC Med	QC Low	LLOQ	QC High	QC Med	QC Low	LLOQ
Nominal concentration (ng/mg)	32.5	16.3	1.56	0.625	32.5	16.3	1.56	0.625
Average measured concentration (ng/mg)	33.3	16.6	1.66	0.674	33.2	16.4	1.62	0.665
n	6	6	6	6	18	18	18	18
STDEV	0.201	0.156	0.0320	0.0170	0.467	0.192	0.0522	0.0441
% CV	0.6	0.9	1.9	2.5	1.4	1.2	3.2	6.6
% Accuracy	102.5	101.9	106.6	107.9	102.3	101.1	103.9	106.5

The intraday % CV was below 3% and the % Accuracy between 101.9% and 107.9%. The interday % CV was below 7% and the % Accuracy between 101.1% and 106.5%. Even at the LLOQ, the % CV and % Accuracy fell well within the acceptable limits. From the validation results shown above for the calibration standards as well as the QCs, it can be concluded that the evaluated method is accurate and precise over the calibration range of 0.625 – 40 ng/mg.

Results from the analysis of authentic QCs also showed good intraday and interday precision, as shown in Table 4.9. The intraday % CV was only 2% and the interday % CV was less than 12%. These results fall within the guidelines to validate spiked QCs and demonstrate that the method has good precision for the measurement of EFV in authentic hair samples.

Table 4.9 Intraday and interday precision of authentic hair quality controls

	Intraday	Interday
Average measured concentration (ng/mg)	5.80	5.61
n	5	15
STDEV	0.113	0.654
% CV	2.0	11.7

4.3.2.4 Matrix effects

The Matuszewski method [216, 217] was used to study possible ion enhancement or suppression effects that can be caused by components within the hair matrix. This method attempts to quantify the effect on the ionisation of the analyte across the calibration range. EFV blank hair obtained from eight healthy volunteers was spiked with EFV at high, medium and low QC concentrations and analysed in order to determine possible matrix effects. The results are shown in Table 4.10 below.

Table 4.10 Peak area ratios of high, medium and low quality controls to determine matrix effects for efavirenz in hair

	High 32.5 ng/mg	Medium 16.3 ng/mg	Low 1.56 ng/mg	Area Ratio vs Concentration Regression Slope
	Peak Area Ratio			
Matrix 1	8.51	3.97	0.432	0.261
Matrix 2	8.27	3.93	0.439	0.253
Matrix 3	8.35	3.95	0.332	0.259
Matrix 4	8.67	4.03	0.351	0.269
Matrix 5	8.56	3.95	0.348	0.266
Matrix 6	8.56	4.04	0.364	0.265
Matrix 7	8.46	3.91	0.342	0.263
Matrix 8	8.65	3.97	0.342	0.269
Average	8.50	3.97	0.369	0.263
STDEV	0.140	0.0461	0.0422	0.00528
% CV	1.6	1.2	11.5	**2.0**

The peak area ratios for each concentration level (high, medium and low) are used to generate regressions for each of the individual matrix sources. The slope variability (% CV) for the different matrix sources should not be greater than 5%. The slope variability for the eight different hair sources was 2.0%, indicating that the precision of this method is not affected by matrix ion enhancement or suppression. This result was expected due to the small amount of hair used, resulting in lower matrix effects.

4.3.2.5 Specificity

Specificity for EFV was evaluated by analysing blank hair samples collected from six healthy volunteers for possible interference between EFV and endogenous substances. A significant interference peak was defined as a peak at the EFV retention time with a peak area greater than 20% of the peak area of the LLOQ. No significant interference peaks were observed for EFV in any of the six blank hair samples analysed. The method is therefore highly specific for the analysis of EFV. A representative blank chromatogram is shown in Figure 4.7.

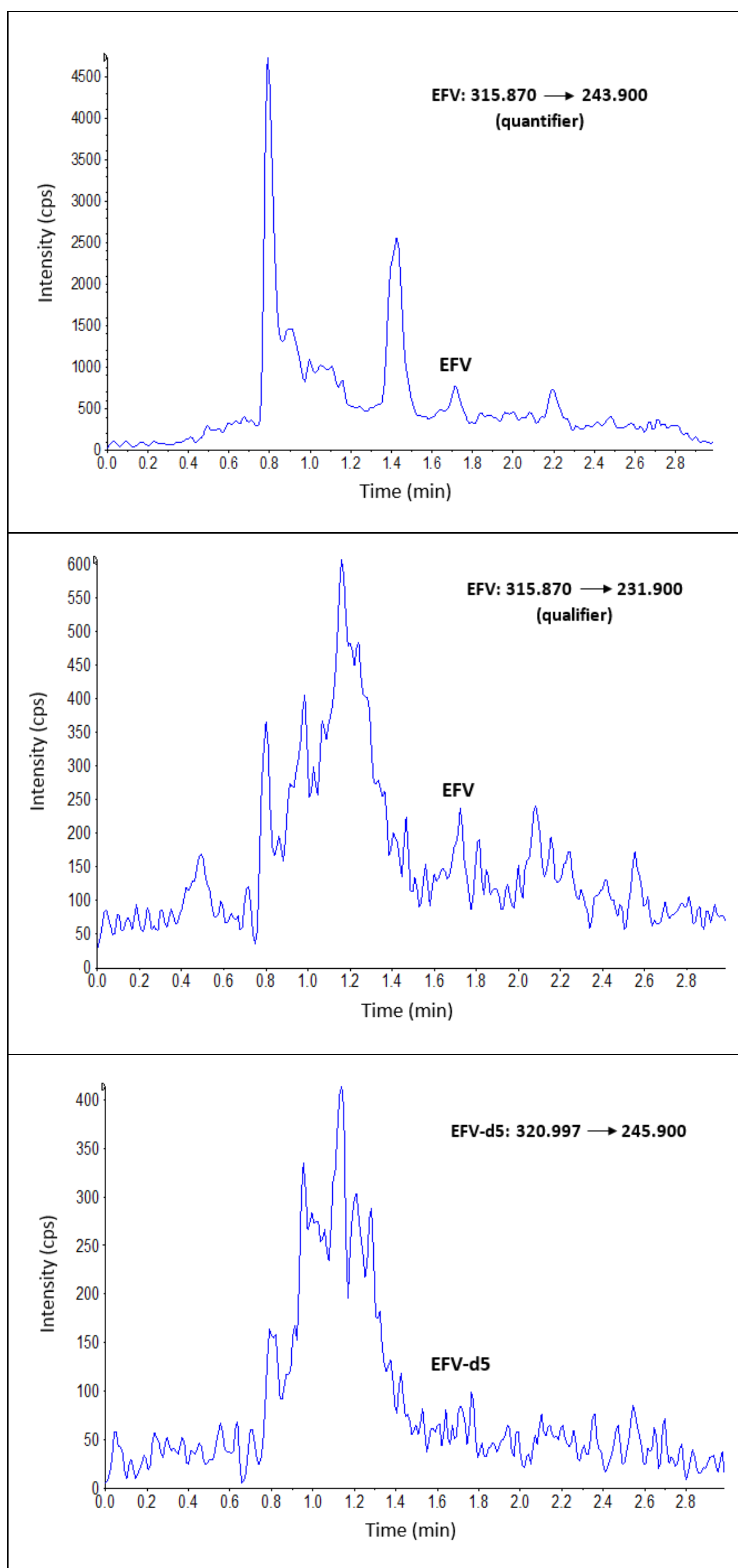

Figure 4.7 MRM chromatograms of a blank hair sample after efavirenz analysis.

4.3.2.6 Sensitivity

The LLOQ of the developed method is 0.625 ng/mg. As shown in Figure 4.8, the S/N ratio for EFV at the LLOQ well exceeds the recommended S/N ratio of 5:1.

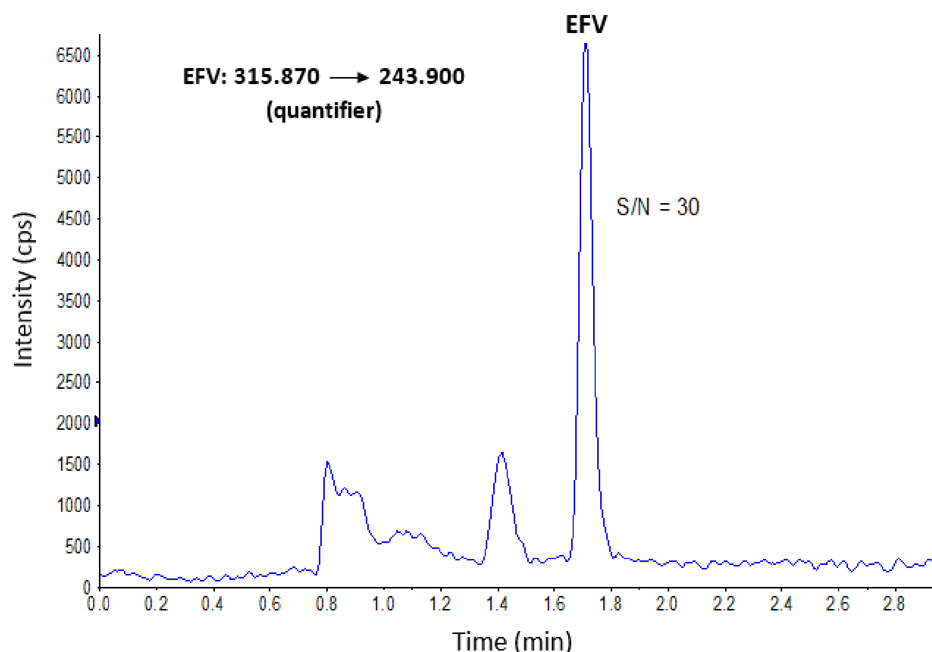

Figure 4.8 Chromatogram of blank hair spiked with efavirenz at the lower limit of quantification (0.625 ng/mg).

A previously validated LC-MS/MS method by Huang et al. [197] reported a LLOQ of as low as 0.05 ng/mg using 2 mg of hair. The LLOQ of 0.625 ng/mg reported in this method was determined using 0.2 mg of hair and is therefore comparable to the LLOQ of 0.05 ng/mg reported by Huang et al. The S/N ratio of 30:1 (shown in Figure 4.8) indicates that a lower LLOQ could have been validated for this method. However, median EFV concentrations in hair reported in literature for patients responding to ART are above 3.0 ng/mg [197, 202, 234]. In patients not responding to ART a median EFV concentration of 0.68 ng/mg [197] has been reported. Therefore, the sensitivity of the validated method is adequate to determine EFV levels in samples of hair collected from HIV-infected patients currently on an EFV-based ART regimen.

4.3.2.7 Carry-over

Carry-over was assessed and monitored during all of the analytical runs by injecting a double blank sample (containing no analyte or internal standard) immediately after the highest calibration standard. Additionally a blank sample (without analyte) was also included to determine any possible contamination of the analyte by the internal standard with an additional carry-over effect. Chromatograms of double blank and blank samples are presented in Figures 4.9 and 4.10.

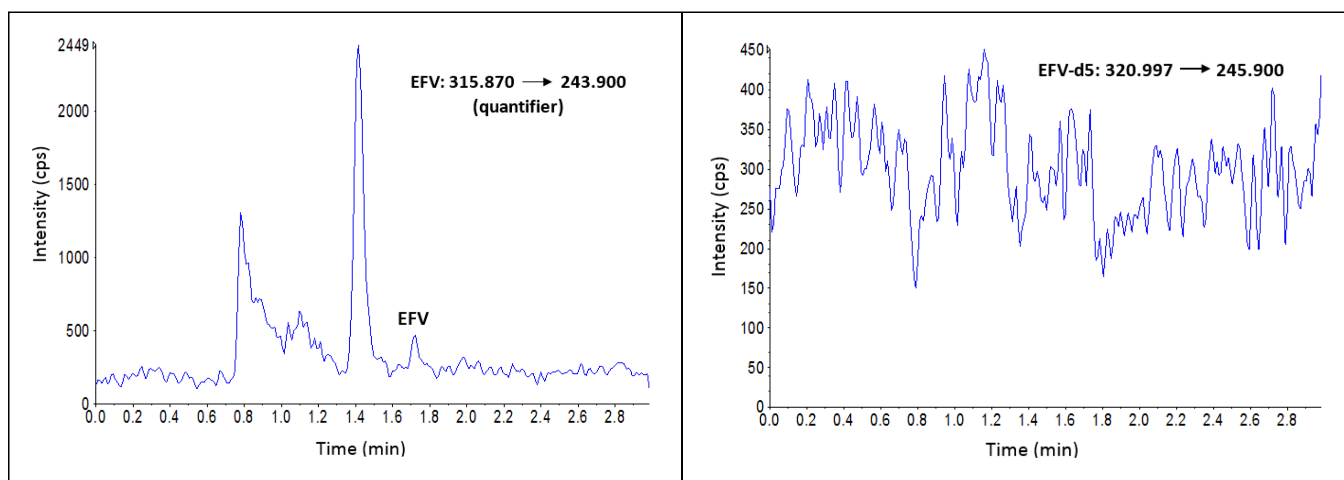

Figure 4.9 Chromatogram of a double blank hair sample. MRM transitions of the quantifying ion for efavirenz (left) and internal standard (right) are shown.

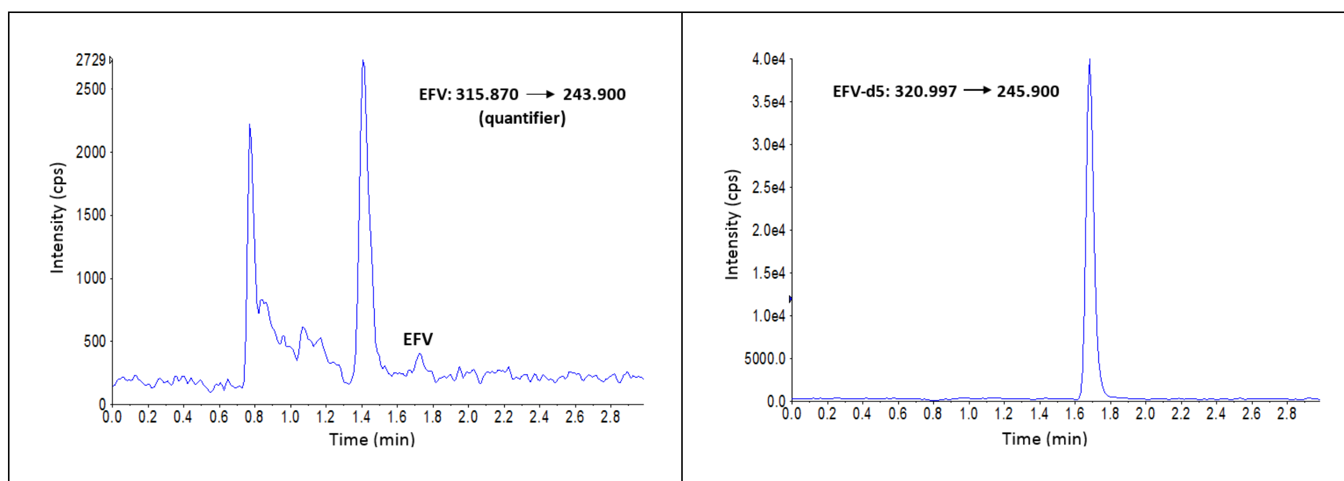

Figure 4.10 Chromatogram of a blank hair sample. MRM transitions of the quantifying ion for efavirenz (left) and internal standard (right) are shown.

From the chromatogram of the double blank hair sample presented in Figure 4.9 it is observed that the double blank sample contains no significant carry-over or contamination. The peak area of the small EFV peak seen in the chromatogram is less than 20% of the peak area obtained at the LLOQ. Similarly, it is observed from the chromatogram of the blank hair sample presented in Figure 4.10 that there is no contamination of the analyte by the internal standard and therefore no additional carry-over effects. The peak area of the small EFV peak seen in the chromatogram is comparable to the peak area of the EFV peak seen in the chromatogram of the double blank sample, and therefore the peak is not a result of contamination by the internal standard.

4.3.2.8 Autosampler stability

High and low QCs extracted in the first validation batch were left in the autosampler and re-injected after approximately 24 and 48 hours in order to evaluate the stability of EFV in processed samples. The peak area ratios of the re-injected QCs were compared to the peak area ratios of the QCs from the first injection. Comparisons of the peak area ratios for the re-injected high and low QCs with the QCs from the first injection are shown in Tables 4.11 and 4.12 respectively. A high % CV and a % difference greater than 15% of the measured values indicates autosampler instability.

The % CV was not greater than 3% for both the high and low QCs after 24 and 48 hour re-injections. Additionally, the % difference was less than 1% for both the high and low QCs after 24 and 48 hour re-injections. These results indicate autosampler stability for up to 48 hours.

Table 4.11 Autosampler stability of extracted quality controls spiked at a high concentration of efavirenz

QC High (32.5 ng/mg)	Validation 1	Re-injection (~ 24 hours)	Re-injection (~ 48 hours)
	Peak Area Ratio		
Injection 1	7.62	7.48	7.46
Injection 2	7.44	7.59	7.57
Injection 3	7.59	7.43	7.57
Injection 4	7.56	7.41	7.65
Injection 5	7.45	7.52	7.64
Injection 6	7.71	7.53	7.63
Average	7.56	7.49	7.59
STDEV	0.104	0.0657	0.0691
% CV	1.4	0.9	0.9
% Difference after re-injection		**-0.9**	**0.4**

Table 4.12 Autosampler stability of extracted quality controls spiked at a low concentration of efavirenz

QC Low (1.56 ng/mg)	Validation 1	Re-injection (~ 24 hours)	Re-injection (~ 48 hours)
	Peak Area Ratio		
Injection 1	0.389	0.377	0.386
Injection 2	0.378	0.385	0.385
Injection 3	0.381	0.409	0.386
Injection 4	0.398	0.385	0.399
Injection 5	0.390	0.398	0.407
Injection 6	0.400	0.391	0.393
Average	0.389	0.391	0.393
STDEV	0.00897	0.0113	0.00899
% CV	2.3	2.9	2.3
% Difference after re-injection		**0.4**	**0.8**

However, these results are valid for samples of hair that have been spiked with EFV and then extracted, rather than authentic samples of hair where EFV is incorporated into the hair matrix. To determine the autosampler stability of authentic hair samples processed for EFV, the above analysis was applied to authentic QCs that were also re-injected after 24 and 48 hours. Comparisons of the peak area ratios for the re-injected authentic QCs with the QCs from the first injection are shown in Table 4.13.

Table 4.13 Autosampler stability of authentic quality controls processed for efavirenz

Authentic QC	Validation 1	Re-injection (~ 24 hours)	Re-injection (~ 48 hours)
	Peak Area Ratio		
Injection 1	1.43	1.41	1.36
Injection 2	1.24	1.22	1.22
Injection 3	1.47	1.45	1.48
Injection 4	1.21	1.24	1.21
Injection 5	1.23	1.24	1.22
Injection 6	1.21	1.22	1.19
Average	1.30	1.30	1.28
STDEV	0.120	0.105	0.115
% CV	9.2	8.1	9.0
% Difference after re-injection		**0.1**	**-1.1**

The % CV and % difference for EFV peak area ratios determined from the analysis of authentic QCs are reported to be within 15% after re-injection of the validation batch. The results from this analysis better represents real hair samples and indicates that samples processed for EFV are stable in the autosampler for up to 48 hours.

4.3.3 Additional experiments

4.3.3.1 Extraction reproducibility

In order to determine the reproducibility of the extraction method when applied to authentic hair, a homogenous sample of authentic hair was extracted and analysed on separate occasions over a period of four weeks. The results presented in Figure 4.11 show that the measured EFV concentrations were consistent over the four week analysis period. This method, therefore, demonstrates good reproducibility and reliability in measuring EFV concentrations in authentic samples of hair.

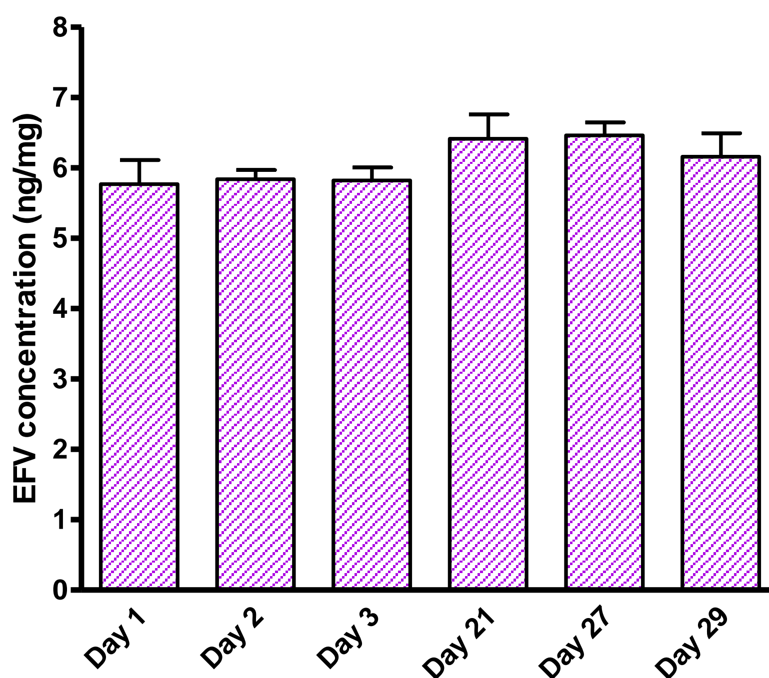

Figure 4.11 Concentration of efavirenz in an authentic sample of hair extracted and analysed on random days over a four week period. Error bars show the standard deviation from the mean value (n = 2). There is no statistical difference between the mean concentrations measured (ANOVA, 95% CI).

4.3.3.2 Extraction efficiency

The extraction efficiency was determined by investigating how much EFV remained in samples of authentic hair after extraction. Authentic hair was extracted three times and

the amount of EFV removed by each extraction was measured. The percentage EFV removed after each of the three extractions is shown in Figure 4.12.

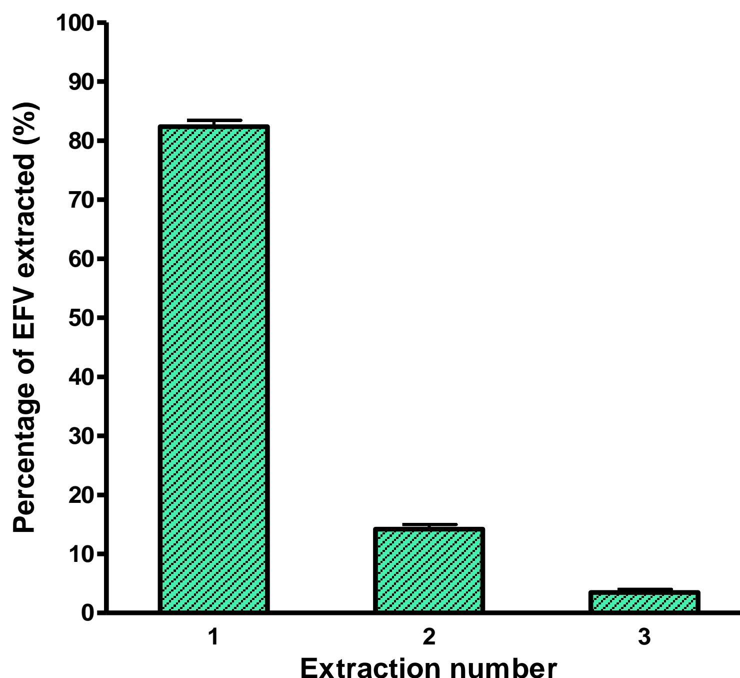

Figure 4.12 Percentage of efavirenz extracted from authentic hair after three repeated extractions, where the total amount of efavirenz extracted was calculated by adding the amount of efavirenz extracted after each of the three extractions. Error bars show the standard deviation from the mean value (n = 6).

The first extraction removed 83% of the total EFV incorporated into the authentic hair. However, this is assuming that all of the EFV was removed after the third extraction. The results indicate that it is likely that a small amount of EFV still remained in the hair and that a fourth extraction might have been necessary to completely remove all of the EFV from the hair. Nonetheless, the results from this experiment indicate that the simple extraction method was effective at removing EFV from a small amount of hair in a short period of time, without the need for an overnight incubation step used in a previously developed method [197].

This approach to investigating the extraction efficiency was first described by Huang et al. [197] for which the percentage of EFV extracted after the first extraction was

estimated to be 95%. This result was determined after two repeated extractions of authentic hair. The extraction method described by Huang et al. [197] was therefore able to remove approximately 10% more EFV from authentic samples of hair compared to the method described in this study. However, the extraction method used in this study is much simpler and less time consuming and therefore more applicable for routine analysis of hair samples.

4.3.4 Analysis of study samples

The validated method for the quantification of EFV in hair was used to analyse the hair samples collected for the broader adherence study (n = 257) in order to monitor the patients EFV levels in hair over the 48 week study period. Figure 4.13 shows the median EFV concentration and IQR for the hair samples collected at weeks 16, 32 and 48 that were above the LLOQ (0.625 ng/mg). The combined median EFV concentration for the hair samples was 5.07 ng/mg (IQR: 2.99 – 8.44 ng/mg), and the combined mean EFV concentration was 7.40 ng/mg (±7.58). A representative chromatogram of one of the analysed study samples is shown in Figure 4.14.

These results compare well to EFV concentrations reported in previous studies. In one study hair samples (n = 134) were collected from South African HIV-infected women and analysed for EFV. The median EFV concentration was reported to be 5.60 ng/mg (IQR: 3.93 – 7.06 ng/mg) [202]. Additionally, mean EFV concentrations in hair of 5.92 ng/mg [233], 6.3 ng/mg [234] and 5.7 ng/mg [191] have been reported for studies conducted outside of South Africa.

As discussed previously in Chapter 1, hair grows at approximately 1 cm per month [57] and as the EFV concentrations were measured in the proximal centimeter of hair, the concentrations reported here represent average drug exposure for the month preceding the time at which the hair samples were collected. However, since the rate of growth of hair varies according to the individual ARV concentrations in hair are only an estimate of the average drug exposure of an individual.

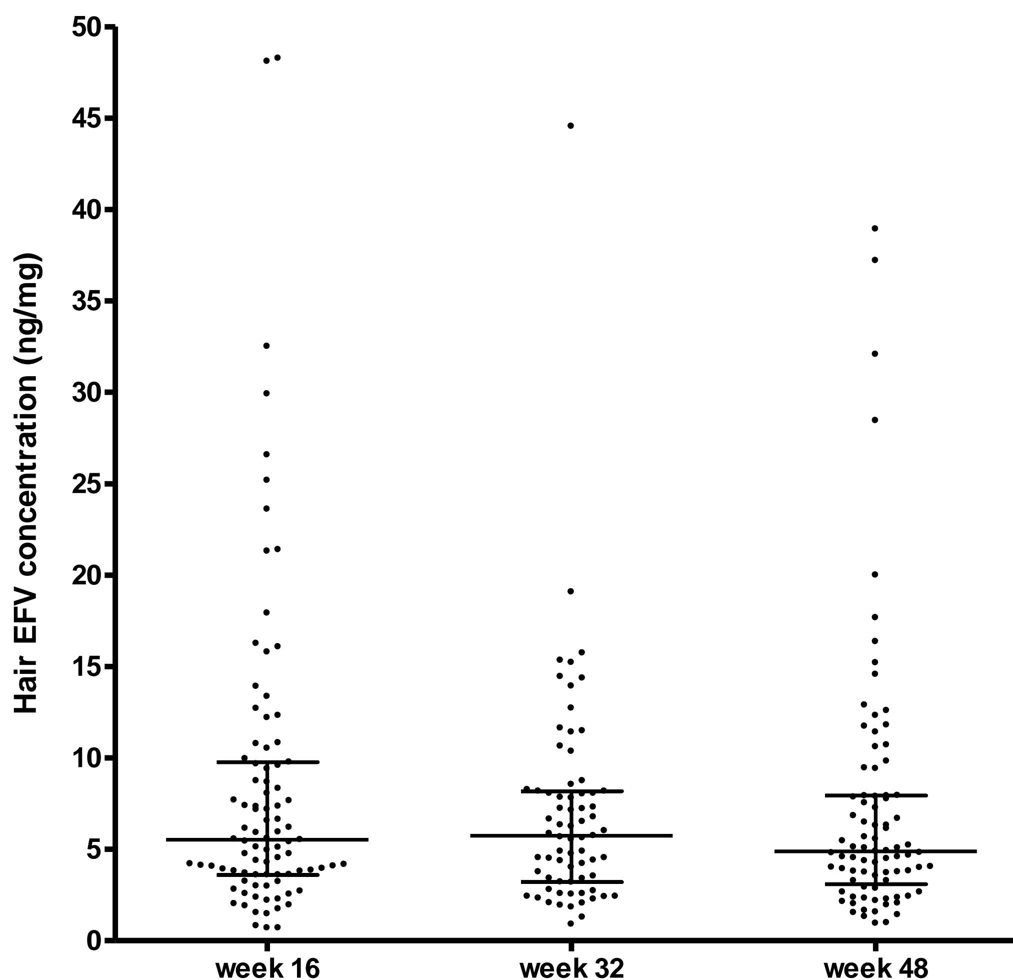

Figure 4.13 Median (interquartile range) efavirenz concentrations for hair samples with levels above the lower limit of quantification at weeks 16 (n = 91), 32 (n = 71) and 48 (n = 85).

Only 10 (3.89%) of the hair samples analysed for EFV gave results that were below the lower limit of quantification (BLQ). In most cases, if one of the hair samples collected from a patient was BLQ then any later hair samples collected from the same patient were also BLQ, indicating that these patients had consistently very low levels of EFV in their circulatory system. Further discussion of the determined EFV concentrations in the context of the broader adherence study will take place in the following chapter.

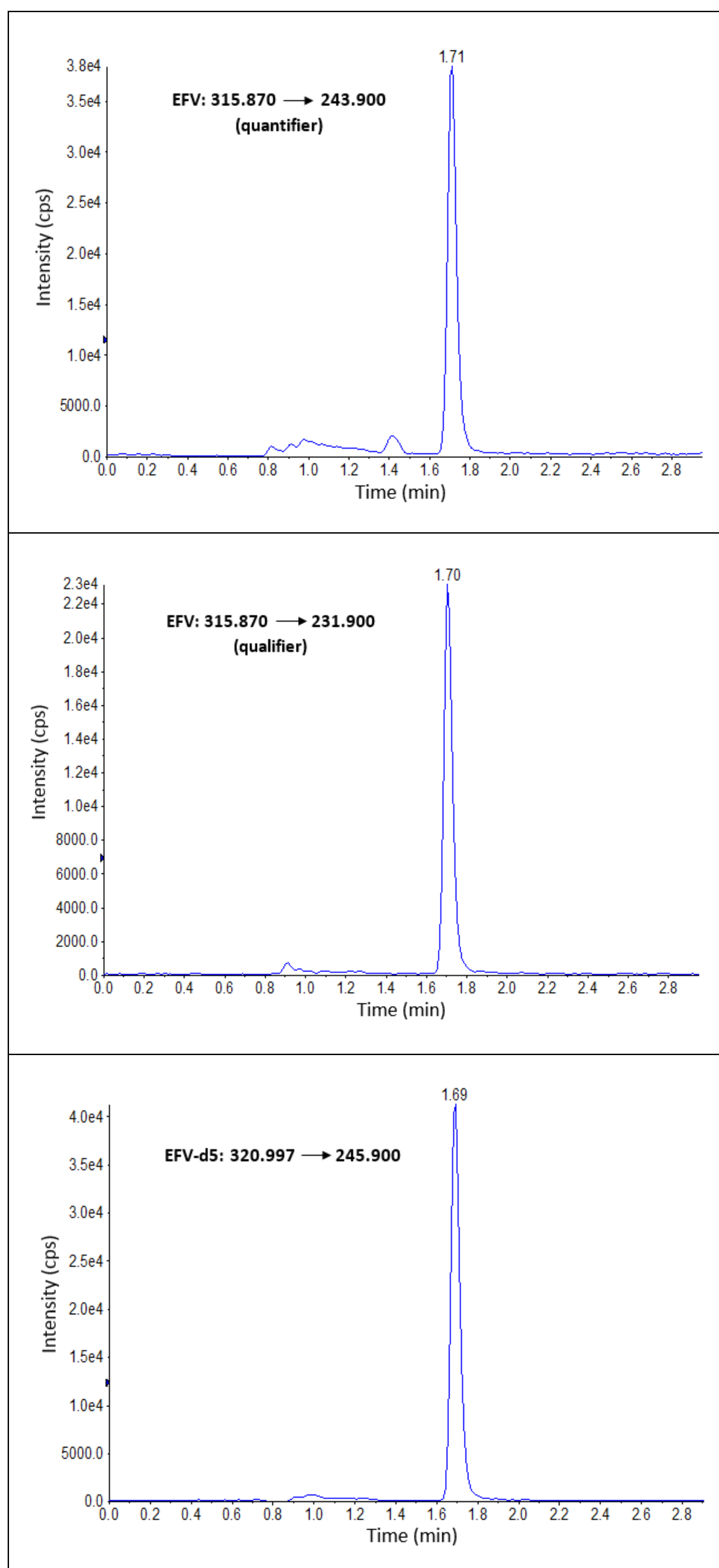

Figure 4.14 MRM chromatograms of an authentic hair sample positive for efavirenz (3.60 ng/mg), spiked with internal standard (12.5 ng/mg).

4.4 Summary and Conclusions

This chapter describes the development and validation of a method for the quantitative determination of EFV in a small amount of hair. EFV was extracted from 0.2 mg hair samples in a short period of time using a simultaneous pulverization and extraction method. This is in contrast to a previously validated method in which EFV was extracted from 2 mg of hair during an overnight incubation step [197].

Despite a simple and quick extraction method, results indicate that 83% of the total EFV incorporated into authentic hair was removed during extraction. However, this result is approximately 10% less than the extraction efficiency achieved by Huang et al. [197] when using an overnight incubation step. Results from investigations carried out by Huang et al. [197] suggest that EFV was maximally extracted from cut hair after 14 hours of incubation. As such, the extraction efficiency of the current method might have improved if a longer incubation time had been incorporated, however, this was not investigated. Although, the reproducibility of the assay is more important than the extraction efficiency, and this was demonstrated by analysing authentic QCs prepared from hair collected from a patient who received ART.

Using 0.2 mg of hair, the LLOQ for EFV was set at 0.625 ng/mg. Based on previously published data, the sensitivity of this method is sufficient to monitor EFV in small amounts of hair collected from HIV-infected patients on EFV-based ART. No significant interference peaks were observed when analysing EFV blank hair collected from six healthy volunteers, indicating that the method is highly sensitive as well as specific for the analysis of EFV. The method was validated over the calibration range of 0.625 – 40 ng/mg and intraday and interday accuracy and precision results for both the calibration standards and QCs fell within accepted criteria.

This method was successfully applied to 257 samples of hair collected from HIV-infected patients. The concentrations of EFV found in the hair samples were comparable to EFV concentrations that have been reported in previous studies [191, 202, 233, 234]. Whilst

these results are significant as they provide insight into EFV levels measurable in hair, the therapeutic range for EFV in hair within the South African context needs to be determined in order to give quantitative results more value within the field of TDM. The EFV concentrations reported in this chapter will be further discussed in the following chapter in the context of reported EFV concentrations in plasma samples as well as other adherence data obtained for the purposes of the broader adherence study.

There are many advantages to the method described in this chapter. The extraction and LC-MS/MS analysis of EFV is not time consuming and results can be obtained within a short period of time after receiving hair samples for analysis. The quick turnover time makes this method appealing for use within routine drug monitoring. Furthermore, only a small amount of hair is needed for the extraction process which is beneficial, especially in situations where limited quantities of hair are available for analysis, as was the case for this study. Finally, this is the first method developed in South Africa for the monitoring of ARV drug exposure using hair as a matrix and provides an alternative approach to conventional TDM methods currently in use.

5 Hair analysis measures in the context of the adherence data

5.1 Introduction

Currently, in South Africa, there are over 7 million people living with HIV. South Africa has a high HIV prevalence at 18.9 % of the adult population. The country also has one of the largest ART programs globally, with approximately 3.7 million HIV-infected individuals receiving treatment in 2016 [1]. For treatment to be successful in preventing disease progression high levels of adherence are essential [16, 18]. Measuring ART adherence is important not only to identify patients in need of interventions, but also to evaluate current interventions aimed at improving adherence. Therefore, accurate measures of adherence are essential in both clinical and research settings [19]. As has been discussed previously, methods for measuring adherence include; patient self-report, pill counts, pharmacy refill records, electronic drug monitoring and TDM which involves the determination of drugs or drug metabolites in plasma, and more recently hair [27] (Chapter 4). However, each of these methods has clear advantages and disadvantages which have been discussed in detail in the literature [16, 18–21], and there is currently no gold standard for determining adherence [15].

A recent study by Orrell et al. [235] compared various adherence measures in an ART-naïve cohort within a resource poor setting in South Africa to determine which adherence measure best predicted virologic and resistance outcomes. Adherence data was collected using self-reports, tablet returns, pharmacy refills, TDM (plasma EFV concentrations), as well as data collected from an electronic adherence monitoring device (EAMD). Results from the study indicated that measures which best predicted virological failure and resistance development were pharmacy refills and EAMD data.

Substance abuse is prevalent among HIV-infected individuals [31] and is also one of the many factors associated with poorer levels of ARV adherence [32]. In South Africa alcohol is the most abused substance, although the use of MA, MQL, cannabis, COC and heroin are still common [226]. Recent studies among South African patients attending HIV clinics have shown hazardous and harmful use of alcohol and/or other drugs to be predictive of poorer adherence [40, 41]. In a meta-analysis by Hendershot et al. [34],

alcohol drinkers were only 50% to 60% as likely to be classified as adherent compared to non-drinkers. This effect was more evident for heavy drinkers than for social drinkers. Heavy drinking is prevalent in South Africa [39] and previous studies have found a negative association between alcohol use and adherence among HIV-infected individuals [43–47]. Research conducted within other low-income settings such as Botswana [236] and West Africa [237] have shown a similar relationship between alcohol use and adherence.

A limitation to the studies mentioned above is that alcohol and/or drug use was determined by self-report and the results are, therefore, subject to recall bias. Self-report screening tools such as the CAGE questionnaire [238, 239], AUDIT [52] and DUDIT [53] have previously been used to identify alcohol and drug problems among HIV-infected patients. In a preliminary investigation conducted among HIV-infected patients attending a clinic in Cape Town, South Africa detection of the biomarkers FAEE and EtG in hair samples were used to verify self-reported alcohol abuse [220]. As this was a pilot study, only 43 participants were recruited. Hair samples were collected and analysed from 30 of the 43 participants. Results from the study indicated that whilst the self-report was highly sensitive, it was not very specific and tended to over predict the likelihood of an individual having an alcohol problem.

While self-reports are valuable screening tools to identify alcohol and/or drug problems, the use of hair biomarkers, as was done in the current study, can be beneficial as an objective measure to confirm self-reported substance use. Therefore, whilst this is not the first study to investigate the relationship between substance abuse and ART adherence in South Africa, it is the first to use objective measures, such as hair analysis for biomarkers, to do so. This chapter aims to analyse the data generated in the previous three chapters in the context of data collected for the broader adherence study in order to investigate the relationship between substance abuse and ART adherence in the study population. Additionally, the use of hair as a matrix for the determination of EFV concentrations as a method of adherence monitoring in the South African context will be investigated.

5.2 Methods

5.2.1 Setting and participants

The parent study was a randomized controlled trial in ART-naïve individuals over 48 weeks which showed that SMS reminders triggered by real-time EAMD had little impact on adherence to ART [240]. Participants were recruited at a large outpatient ART centre in Gugulethu, Cape Town; the Hannan Crusaid Treatment Centre (HCTC). ART-naïve adults and adolescents (≥ 15 years old) were eligible for the parent study if they were commencing treatment at the HCTC, had their own mobile phone and were willing to sign an informed consent form. The details of the parent study have been described elsewhere [240].

5.2.2 Sub-study design and participants

Participants also had the option of joining a non-randomised voluntary sub-study to understand the impact of objectively measured substance abuse on adherence and response to ART. Participation involved providing samples of hair at weeks 16, 32 and 48. In order to minimise inconvenience sub-study visits were arranged to coincide with booked clinic visits. Of the 230 individuals enrolled into the parent study, 135 provided hair samples. The majority of this subset cohort was female (93%) from the South African Black (SAB) population. A total of 257 hair samples were collected from the 135 individuals, consisting of 93 at week 16, 75 at week 32 and 89 at week 48. On average two hair samples were collected from each of the individuals who participated in the sub-study. This chapter analyses data for the subset of 135 participants who provided hair samples.

5.2.3 Measures and analyses

The measures collected for the parent study [240] as well as an additional voluntary pharmacokinetic and pharmacogenetic sub-study [241] that were relevant to the present study are described on the following page.

In the parent study adherence was monitored using a Wisepill® device [242], a real-time EAMD shown in Figure 5.1. The Wisepill® device is the size of a mobile phone and can store up to a week of medication in a seven compartment pill box. Every participant received a Wisepill® device and each time the device was opened a signal was sent via the mobile phone network to a secure central computer, thereby recording tablet taking or treatment interruptions in real time. Any recorded opening on a day during the study was classified as an adherent day, and cumulative adherence was calculated as the number of adherent days divided by the number of days in care.

Figure 5.1 Wisepill® electronic adherence monitoring device [242].

To screen participants for alcohol problems, the CAGE questionnaire was completed by participants at screening and at weeks 16 and 48. The CAGE questionnaire consists of only the following four questions:

Have you ever felt you should **C**ut down on your drinking?

Have people **A**nnoyed you by criticizing your drinking?

Have you ever felt bad or **G**uilty about your drinking?

Have you ever had a drink first thing in the morning to steady your nerves or get rid of a hangover (**E**ye opener)? [238, 239]

Two or more positive answers identifies participants with alcohol problems [243, 244]. Whilst participants were screened specifically for alcohol problems, no screening tool was used to screen participants for drugs of abuse.

Blood was drawn for HIV-1 viral load (HIV-1 RNA 3.0 assay®; Bayer Healthcare, Leverkusen, Germany) at screen and at weeks 16 and 48. Additional blood was drawn for mid-dosing interval EFV concentrations (in the window between 9 and 16 hours after self-reported efavirenz intake time) at weeks 16, 32 and 48 and CYP2B6 pharmacogenetic analysis. For the determination of EFV concentrations blood samples were centrifuged at 3500 rpm for 10 min. Plasma was transferred into labelled cryovials that were frozen at -80°C until analysis. Plasma EFV concentrations were determined by the Division of Pharmacology, Department of Medicine, University of Cape Town using a LC-MS/MS method validated for the range 0.0195 – 20 µg/ml. The pharmacogenetic analysis of the blood samples is described in detail elsewhere [241]. Briefly, blood samples were centrifuged at 3000 rpm for 30 min and the white blood cell layer transferred into a labelled cryovial and frozen at -80°C until analysis. Three CYP2B6 single nucleotide polymorphisms previously associated with EFV concentrations were chosen and analysed. Based on their genotype participants were classified as either slow, intermediate or extensive metabolisers using a simplified version of Holzinger et al.'s [245] metaboliser status classifications.

Hair samples, collected at weeks 16, 32 and 48, were analysed for EtG and drugs of abuse using validated LC-MS/MS methods (described in Chapters 2 and 3) in order to determine the prevalence of substance abuse within this subset. Additionally, hair samples were analysed for EFV using a validated LC-MS/MS method (Chapter 4) to investigate the possibility of using EFV concentrations in hair as a measure of adherence.

5.2.4 Statistical analysis

GraphPad Prism 4 (California, USA) was used for the statistical analysis of data. Samples that were previously determined to be BLQ were analysed as 0.624 ng/mg for EFV concentrations in hair and 0.0194 µg/ml for EFV concentrations in plasma.

5.2.5 Ethical approval

Ethical approval for the study was given by the University of Cape Town, Faculty of Health Sciences, Human Research Ethics Committee. Informed consent was provided by each of the study participants. The broader clinical trial was registered in the Pan African Clinical Trials Registry (number PACTR201311000641402).

5.3 Results and Discussion

5.3.1 Cohort adherence

The adherence of the cohort, comprised of the 230 individuals randomised to the parent study, and the subset, comprised of the 135 individuals who gave hair samples to be analysed for the current study, are compared in Table 5.1 below.

Table 5.1 Cumulative Wisepill® adherence at weeks 16 and 48 for the cohort and subset

	Week 16		Week 48	
	n	**Adherence (%): median (IQR)**	**n**	**Adherence (%): median (IQR)**
Cohort [235]	160	93 (74-98)	180	86 (59-94)
Subset	92	100 (92-100)	89	101 (96-107)

As shown in Table 5.1 high levels of adherence were observed in the subset and the median adherence of the subset was higher at both weeks 16 and 48 compared to that of the cohort. This indicates that the hair samples analysed in the current study were provided by participants who were retained in care and likely to be taking their ARVs on a daily basis. In addition, a high percentage of these participants were virally suppressed. At week 16, only 4 (4.3%) of 92 participants were not virally suppressed (viral load > 400 copies/ml), and at week 48, only 5 (5.6%) of 89 participants were not virally suppressed (viral load > 50 copies/ml). Due to the low occurrence of virological failure, the association between variables and virologic outcomes was not assessed in this current

study. Wisepill® adherence data provided more variability and was therefore used instead. Moreover, Wisepill® adherence data collected from the cohort was previously shown to best predict virological failure and resistance development when compared to other adherence measures [235].

5.3.2 Sensitivity and specificity of the CAGE questionnaire

The results from the CAGE questionnaire identified only 13.5% of participants at week 16 and 7.9% of participants at week 48 as having an alcohol problem. However, results from the analysis of hair samples for the alcohol biomarker, EtG, identified 29.2% of participants at both weeks 16 and 48 as heavy drinkers. When analysing the results from both weeks 16 and 48 the sensitivity and specificity of the CAGE questionnaire for this study were calculated to be 28.8% and 96.8% respectively, if hair analysis is considered to be the gold standard. Therefore, whilst the CAGE questionnaire was accurate in detecting participants who did not have an alcohol problem, indicated by the high specificity of the questionnaire, it missed a large majority of participants who did have a problem with alcohol, indicated by the low sensitivity of the questionnaire.

Although the CAGE questionnaire has previously been shown to be a useful screening tool to identify alcohol problems among HIV-infected patients entering medical care [243], the results from the CAGE questionnaire in the context of this study largely underestimated the number of participants with alcohol problems according to the results from the analysis of hair for EtG.

5.3.3 Substance abuse and adherence

5.3.3.1 Prevalence of substance abuse

The prevalence of heavy drinking in the subset for the duration of the study was 27%, whilst the prevalence of chronic drug use was 5.9%. Just over half (51.49%) of the participants who consumed alcohol were heavy drinkers, supporting the fact that heavy drinking is prevalent among South Africans [39]. A similar finding was reported in a study

by Morojele et al. [43] on HIV-infected patients where 53.3% of drinkers were classified as heavy drinkers.

In a systematic review of studies conducted in sub-Saharan Africa related to depression, alcohol use and ART adherence, the prevalence of harmful use of alcohol among study participants ranged from 7 – 31% [42]. However, studies conducted in Cape Town, South Africa have reported higher rates of harmful alcohol use among people living with HIV/AIDS (PLWHA). One such study reported the prevalence of harmful alcohol use to be 37% and the prevalence of problematic drug use to be 13% [40], whilst another reported a prevalence of 46% and 15% respectively [41]. Both of these studies reported significant gender differences in both the harmful use of drugs and alcohol with males being significantly more likely to engage in the harmful use of drugs and alcohol. The lower prevalence of substance abuse reported in this study is most likely due to the predominantly female subset, although it is also possible that the reported prevalence of heavy drinking is high for a female population. The differences in substance abuse could also be due to the different measuring approaches used as for both of the studies discussed above substance abuse was measured by self-report (AUDIT and DUDIT).

5.3.3.2 Impact of chronic drug use on adherence

The median Wisepill® adherence for the chronic drug users at weeks 16, 32 and 48 were 92% (IQR: 64 - 104), 87% (IQR: 75 -90) and 106% (IQR: 103 – 117) respectively. At weeks 16 and 48 there was no significant statistical difference between the median adherence for those who were chronic drug users and those who were not. At week 32, however, there was a small significant difference between the median adherences of the two groups (Mann Whitney test, $P < 0.05$) which suggests that the adherence of participants who were identified to be chronic drug users was negatively impacted, possibly due to chronic drug use during this time. However, the low prevalence of chronic drug use does not allow for the impact of chronic drug use on adherence to be investigated further.

5.3.3.3 Impact of heavy drinking on adherence

The median Wisepill® adherence (%), with IQR, for the participants in the subset classified as heavy drinkers compared to those classified as social and non-drinkers is represented below in Figure 5.2.

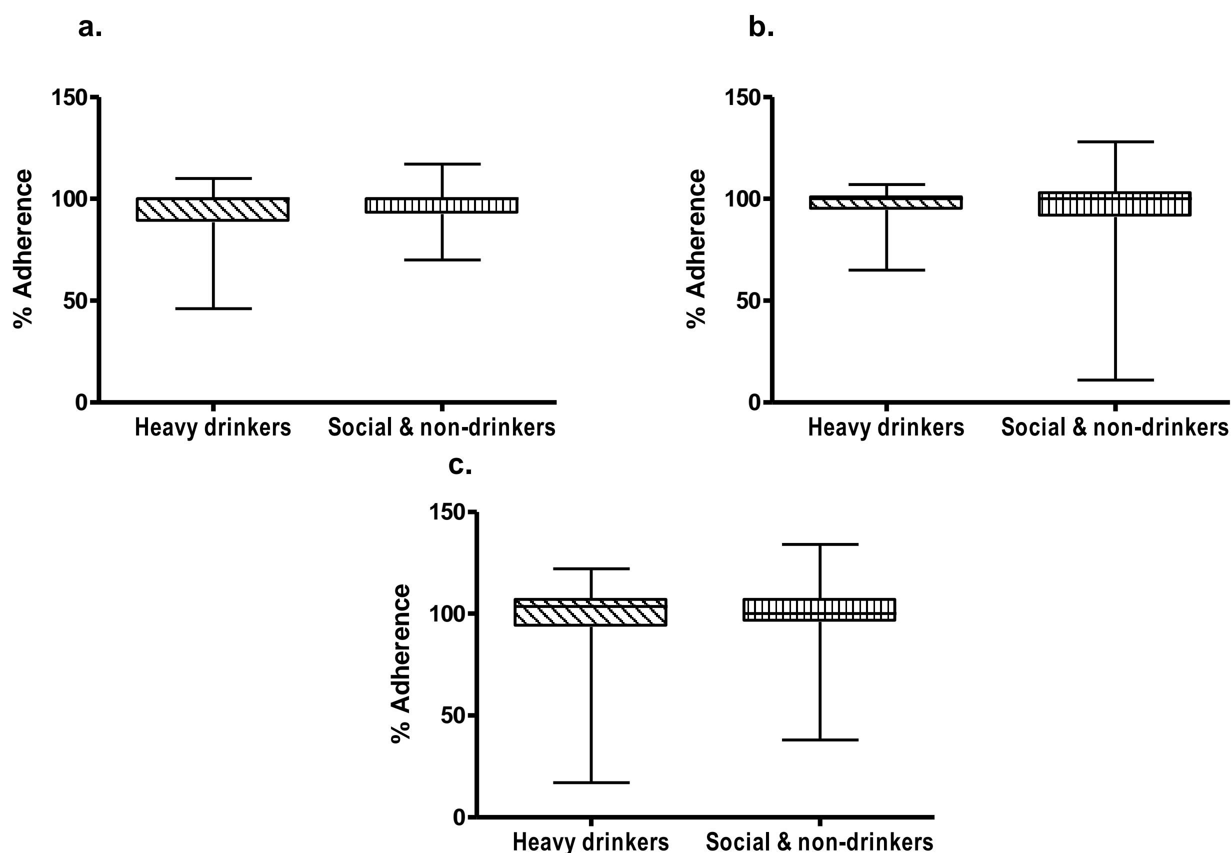

Figure 5.2 Median percentage adherence with interquartile range for heavy drinkers and social and non-drinkers at weeks 16 (**a**), 32 (**b**) and 48 (**c**).

The median adherence for the heavy drinkers did not differ significantly from the median adherence for the social and non-drinkers at weeks 16, 32 or 48. These results suggest that, despite nearly a third of the subset being classified as heavy drinking, it did not impact on adherence. The lack of association between heavy drinking and adherence was unexpected as previous studies investigating the impact of alcohol use on adherence in sub-Saharan Africa have reported heavy drinking to be associated with adherence [40, 41, 46, 236, 237, 246]. Although, similar to the results reported in this

study, a study conducted among HIV-infected patients attending a clinic in Nigeria also reported no association between heavy drinking and poor adherence [247].

The most likely reason for the lack of association between heavy drinking and adherence observed in this study is that the majority of participants who were prepared to provide hair samples were those who were retained in care and did not experience virologic failure. This indicates that even the participants who were heavy drinkers were also adherent, taking their ARVs on a daily basis. Additionally, the majority of this subset was female as in most cases male participants did not have any scalp hair that could be collected. Not only is adherence generally better in females compared to males in sub-Saharan Africa [40, 41, 248], but most studies report a higher prevalence of substance abuse (chronic drug use as well as alcohol abuse) in male participants compared to females [40, 41, 47, 236]. Therefore, this investigation into the impact of heavy drinking on adherence was limited by the subset being predominately comprised of females who were good at taking their ARVs, even in instances of heavy drinking.

5.3.4 Relationship between efavirenz concentrations in hair and plasma

Scatterplots of the correlation between hair and plasma EFV levels are presented in Figure 5.3. Spearman correlation coefficients were used to assess the relationship between concentrations of EFV in the two matrices. The results indicate that hair and plasma EFV levels were strongly correlated throughout the study (correlation coefficients, 0.672 – 0.741, all P values < 0.0001).

These results suggest that, for this subset, a single plasma concentration was as good an adherence measure as a single hair concentration. This is interesting as hair concentrations provide an average level of drug exposure over time (for example, the last 30 days) [249], whereas single plasma concentrations represent a brief snapshot of drug exposure and are also subject to ‘white coat effects’ [26]. Therefore, it is suggested throughout literature that hair ARV concentrations might be of more value than single plasma concentrations [27, 201, 249]. However, the results from this study suggest that,

for this subset, this was not the case. The strong correlation observed between the hair and plasma EFV levels can be explained by considering the high level of adherence demonstrated by this subset. It is likely that the single plasma concentrations were representative of the average good adherence patterns of this subset which would have also been represented by the hair concentrations.

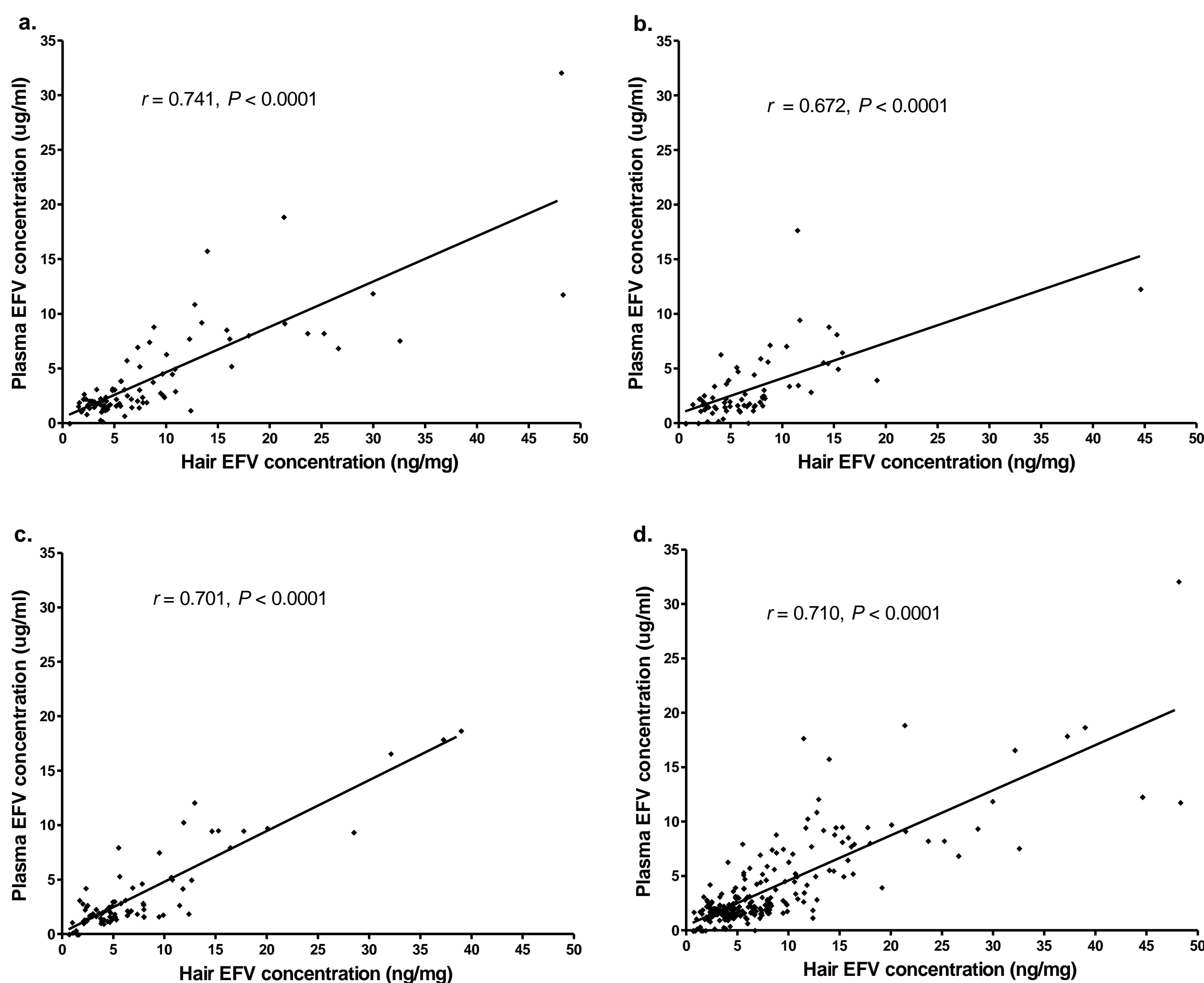

Figure 5.3 Scatterplots showing the correlation between plasma and hair efavirenz concentrations at weeks 16 (**a**), 32 (**b**), 48 (**c**) and for all weeks combined (**d**). Spearman correlation coefficients used to assess the relationship between efavirenz levels in hair and plasma are shown.

There is little published data comparing EFV concentrations in hair and plasma, however, in one other study concentrations of EFV in hair were reported to be strongly correlated with 24 hour intensive pharmacokinetic measurements and only weakly correlated with single plasma measurements [190]. On the other hand, studies focussed on other ARVs have found hair concentrations to correlate well with plasma levels [185, 194].

5.3.5 Hair efavirenz concentrations according to metaboliser status

As previously mentioned, EFV concentrations in plasma and hair are known to be influenced by CYP2B6 polymorphisms [9, 233]. Since the genotype for the participants was previously determined as part of a related sub-study [241], the EFV levels in the hair samples were analysed according to metaboliser status. Out of the 135 participants who provided hair samples 34 (25.2%) were extensive metabolisers, 61 (45.2%) were intermediate metabolisers and 35 (25.9%) were slow metabolisers. The metaboliser status for five of the participants was missing. The results from the analysis are presented in Figure 5.4.

There was a significant difference between the median EFV concentrations in hair for each metaboliser status (ANOVA, 95% CI, $P < 0.0001$). Median EFV concentrations for the extensive, intermediate and slow metaboliser genotypes were 3.54 ng/mg (IQR: 2.35 – 4.59), 5.11 ng/mg (IQR: 2.93 – 7.94) and 10.66 ng/mg (IQR: 7.01 – 15.93) respectively. A similar result was reported when the corresponding plasma samples, collected for the related sub-study, were analysed according to metaboliser status [241]. Therefore, participants in the cohort with the slow metaboliser genotype displayed significantly higher median EFV concentrations in both short-term and long-term EFV exposure. This observation is consistent with results reported in an earlier study where individuals with the slow metaboliser genotype were shown to have > 3 fold increases in EFV concentrations in both plasma and hair samples [233].

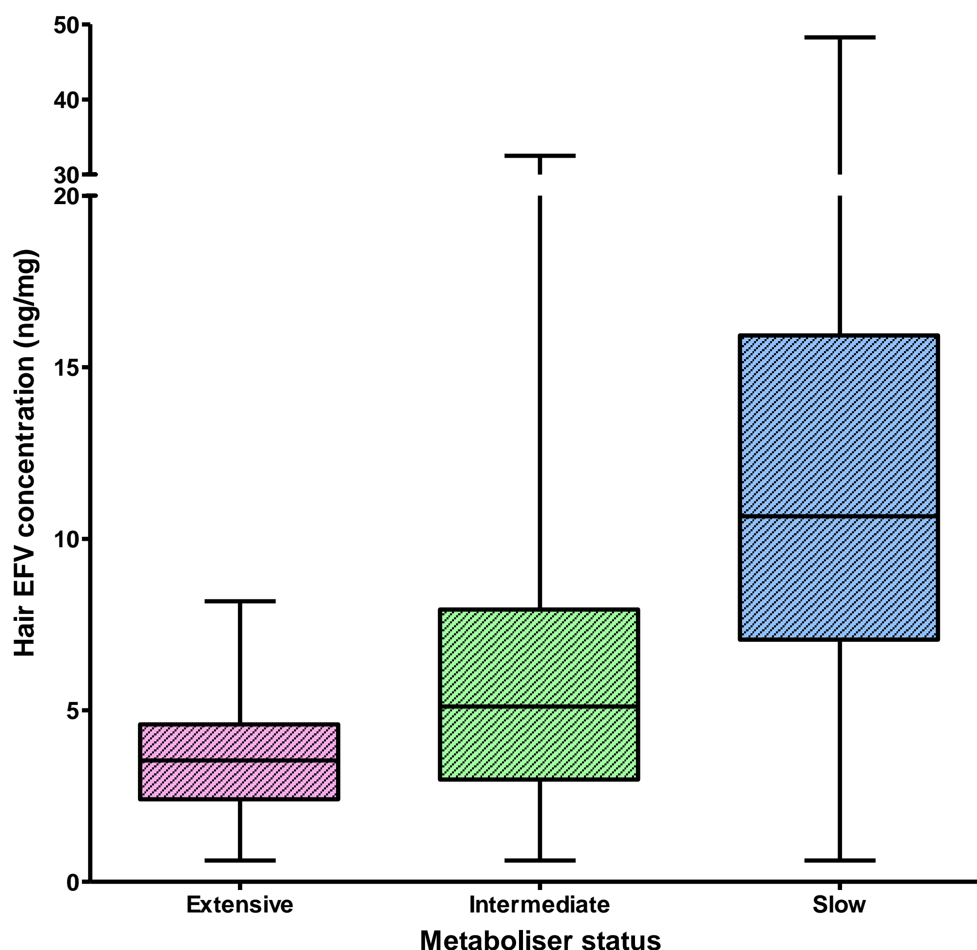

Figure 5.4 Median (interquartile range) efavirenz concentrations in hair according to metaboliser status. Individual concentrations determined at weeks 16, 32 and 48 have been combined for the above analysis.

Besides this current study, one other study has investigated the influence of CYP2B6 polymorphisms in hair samples collected from HIV-infected women in South Africa [202]. The results also showed increased levels of EFV in hair samples from individuals with the slow metaboliser genotype. The study also found no significant association between the CYP2B6 metaboliser genotypes and virologic response, which is consistent with previous studies conducted using plasma samples [241].

5.3.6 Hair efavirenz concentrations as a predictor of adherence

The relationship between Wisepill® adherence and EFV levels in hair was assessed using scatterplots and Spearman correlation coefficients. As can be seen by the results presented in Figure 5.5 no significant correlation between adherence and EFV levels in hair was observed. Although the median Wisepill ® adherence was 100% at weeks 16 and 32 (IQR: 92 – 100 and 92 – 103 respectively) and 101% (IQR: 96 – 107) at week 48, the concentration of EFV in the hair samples varied, most likely due to the different metaboliser genotypes present in the subset.

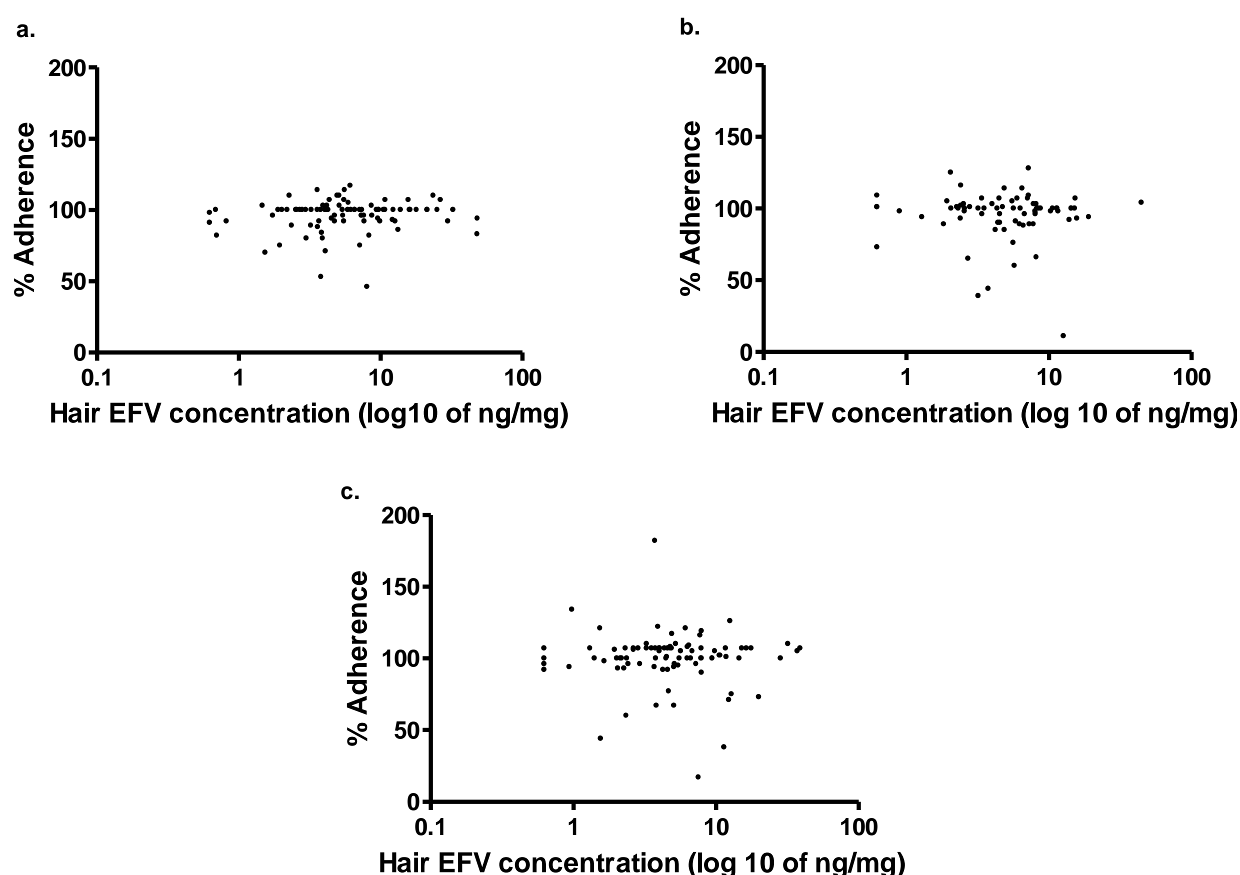

Figure 5.5 Scatterplots showing the relationship of Wisepill® adherence (represented as a percentage) to concentrations of efavirenz in hair at weeks 16 (**a**), 32 (**b**) and 48 (**c**).

Even though previous studies have shown hair concentrations of ARVs to be strong predictors of virologic outcomes [185–187, 189, 193, 201], the small occurrence of virological failure experienced in this subset did not allow for hair EFV levels to be analysed in the context of virologic outcomes. However, studies that have previously

investigated the relationship between self-reported adherence and hair ARV concentrations have reported a weak correlation between the two measures [190, 250]. Olds et al. [251] assessed the use of ARV levels in hair for evaluating ART adherence among HIV-infected children in Uganda. Hair concentrations were compared to other adherence measures, including a Medication Event Monitoring System (MEMS), a type of electronic adherence monitoring system similar to the Wisepill®. Similar to the results reported in this study drug concentrations in hair were found to have a non-significant association with the other adherence measures, including MEMS data.

5.4 Summary and Conclusions

The objective measures of substance abuse and ART adherence determined in the previous three chapters were analysed in this chapter in the context of data collected for the broader adherence study. A comparison between the results from the CAGE questionnaire used to screen participants for alcohol problems and the analysis of hair samples for EtG highlighted the poor sensitivity of the CAGE questionnaire in identifying participants who were heavy drinkers. According to the results from the analysis of hair samples for EtG and drugs of abuse, the prevalence of heavy drinking and chronic drug use in this subset were reported to be 27% and 5.9% respectively.

The prevalence of chronic drug use was too low to make any accurate predictions of the impact that chronic drug use might have had on adherence. However, when comparing the median adherence for heavy drinkers with social and non-drinkers no significant difference was found. Therefore, unlike previous studies that have investigated the impact of alcohol on adherence, the results of this study indicate that despite a high prevalence of heavy drinking within the subset, participants still maintained a high level of adherence.

The strong correlation between the levels of EFV in plasma and hair observed in this study suggests that a single plasma measurement might be as valuable in the context of

adherence monitoring as a hair level. However, the subset for which plasma and hair levels were correlated demonstrated high levels of adherence (Table 5.1) and as such this suggestion is likely only true in the case of patients that demonstrate consistently high levels of adherence. The benefit of analysing both plasma and hair samples for adherence monitoring is that a low plasma concentration indicates recent poor drug exposure whereas a low hair concentration indicates average poor drug exposure for the previous month [185]. Additionally, a high plasma concentration in combination with a low hair concentration could provide insight into the adherence patterns of patients and identify cases where ARVs are being taken just prior to clinic visits.

Whilst hair ARV levels have previously been shown to correlate well with virological suppression [185–187, 189, 193, 201], this has not been the case when the relationship between hair levels and measures of adherence such as self-report and EAMD have been assessed [190, 250, 251]. In this study hair EFV levels were found to have a non-significant association with adherence measured by the Wisepill® device. The low occurrence of virological failures in this subset did not allow for hair EFV levels to be associated with virologic outcomes. The relationship between hair EFV levels and virologic outcomes needs to be further explored in a cohort with a higher rate of virological failures.

The study was limited by the study design in that the participants who provided hair samples were predominantly female, had higher levels of adherence compared to the rest of the cohort and were mostly virologically suppressed. This limited the extent to which the data could be analysed and did not allow for certain associations to be investigated. The collection of hair samples was also challenging and only 135 (59%) of the 230 participants that were enrolled into the parent study provided hair samples. Despite these limitations and challenges, the results presented and discussed in this chapter illustrate the potential of hair analysis to be used in South Africa within the contexts of determining substance abuse and ART adherence monitoring.

6 Conclusions and Future work

Hair analysis for drugs is new to South Africa. Even though this method of measuring drug levels is currently well-established in many countries, hair analysis has only very recently been introduced in South Africa. The methods presented in this study are some of the first hair analysis methods to be developed and validated within the country. These methods have the potential to be used in a variety of different contexts and will, in the future, be used in various other studies. For the purposes of this current study the methods were used to analyse hair samples collected from HIV-infected patients attending a clinic in Cape Town in order to measure substance abuse and ARV adherence. Not only is HIV a major health challenge in the country, but substance abuse is prevalent among HIV-infected individuals [31]. Self-report is commonly used to measure both substance abuse and adherence, however this method of measurement is subject to bias. Moreover, the use of different self-report measures makes it difficult to standardise and compare results between studies. The hair analysis results presented in this study provide objective measurements of substance abuse and adherence among HIV-infected patients.

Alcohol use can be assessed by measuring levels of the ethanol metabolite, EtG, in samples of hair. In this study a quantitative LC-MS/MS method was successfully developed and validated to identify heavy drinking among HIV-infected patients. The simple extraction procedure was effective at removing EtG from hair over a short period of time. However, with an LLOQ of 7.50 pg/mg the method lacks the sensitivity of most previously published methods [74], and in the future it will be necessary to improve the method sensitivity. Nevertheless, the sensitivity of the method is more than adequate to confidently identify cases of heavy drinking based on levels of EtG in hair. Analysis of the samples of hair collected for the study indicated that just over a quarter (27%) of the subset were heavy drinkers.

For this study heavy drinking was identified using the cutoff of ≥ 30 pg/mg EtG in hair proposed by the SoHT [75]. However, this cutoff might not be the most appropriate cutoff to use within the South African context. African hair grows at a slower rate compared to Caucasian hair [252, 253], which could result in generally higher levels of

EtG being detected in African hair samples. Therefore, a slightly higher cutoff value might be more appropriate to identify heavy drinking, however this needs to be investigated. In the future, cutoffs to assess both abstinence and heavy drinking among South Africans, more specifically among the African and Coloured populations, need to be determined.

A preliminary investigation into the washout effect demonstrated that up to 64% of EtG was lost from hair samples following three successive washes with water. Whilst the effect of the use of cosmetic hair treatments on EtG levels was not investigated in this study, previous studies have shown a significant reduction of EtG levels in hair that has been cosmetically treated [78, 116–119], suggesting that EtG is more easily washed out from hair that has been damaged by cosmetic treatments. A concerning implication of this is the increased possibility of reporting false negative results when analysing hair that has been cosmetically damaged. African hair tends to be very curly and as a result nearly two thirds of African females in Cape Town use chemicals, commonly referred to as 'relaxers', to straighten their hair. The use of hair relaxers has been associated with fragile and damaged hair [254]. The effect that using hair relaxers has on EtG levels in hair has not yet been investigated. Therefore, in the future, it will be important to investigate the impact that African hair treatments, such as relaxers, have on EtG levels in hair in order to assist with accurately interpreting hair analysis results and, where possible, to avoid reporting false negative results.

A qualitative LC-MS/MS method was developed and validated to identify chronic drug use among the subset. Even though there were some limitations to this investigation, the results provided insight into the patterns of drug use among these HIV-infected patients. Evidently, alcohol was the most commonly abused substance as only 8 of the 135 participants (5.9%) were identified as positive for chronic drug use, based on the cutoff concentrations recommended by the SoHT [56]. The most commonly detected drug of abuse was MQL, which was present in 64% of the samples reported as positive for chronic drug use. Additionally, more than half (62.5%) of the participants identified to be chronic drug users were also heavy drinkers.

However, for this screening method to be used to its full potential within the country, in the future, it will be necessary to increase the sensitivity of the method as well as to include a wider range of drugs in the screen, specifically drugs that are unique within the South African context. Furthermore, future work will require investigating the effects of African hair treatments on drug levels in hair as well as the role that racial bias has on drug levels, since previous studies have demonstrated increased drug concentrations in non-Caucasian hair [141]. As a result, cutoff levels relevant to the South African context will need to be established.

The objective measures of alcohol and drug use obtained in the current study were used to investigate the relationship between substance abuse and adherence among HIV-infected patients. Whilst the results provided insight into the prevalence of substance abuse within the subset, substance abuse appeared to have no impact on the adherence levels, which was unexpected. Various circumstances surrounding the study, such as the generally high adherence rates observed within the subset, did not allow for the relationship between substance abuse and adherence to be fully explored. Nevertheless, these methods of hair analysis are now available to be used in future studies to, hopefully, provide more accurate measurements of substance abuse among HIV-infected individuals compared to self-report measures that are commonly used within this context. In order for interventions aimed at reducing substance abuse among HIV-infected individuals to be effective, patterns of substance abuse as well as the relationship between substance abuse and adherence among HIV-infected populations in the country need to be well-documented.

A novel method of measuring adherence to ARVs in South Africa was introduced and implemented in this study. A quantitative LC-MS/MS method was developed and validated for the analysis of EFV in hair. Currently, there is only one other method that has been published for the determination of EFV concentrations in hair [197]. The method developed in the current study requires only 0.2 mg of hair and the simple extraction procedure was effective at removing EFV from hair over a very short period

of time using a simultaneous pulverization and extraction step. The simple and quick extraction procedure along with the short LC-MS/MS run time makes this method ideal for the routine analysis of hair samples. The method was applied to the 257 hair samples that were collected for the study in order to monitor the patients' EFV levels in hair. Whilst the measured EFV hair concentrations were comparable to concentrations reported in previous studies [191, 202, 233, 234], the therapeutic range for EFV levels in hair is yet to be determined. In order to give quantitative results more value within the field of adherence monitoring, it will be important for future studies to determine the therapeutic range of EFV in hair collected from South African patients. This was not possible in the current study due to the small number of virological failures experienced in the subset.

A significant correlation was observed between hair and plasma EFV levels, indicating that both the long-term and short-term adherence patterns of this subset were consistently good. Whilst it is unlikely that hair will replace plasma as a matrix for TDM in South Africa, the complimentary use of both matrices in the future will be beneficial in that more information regarding the adherence patterns of individuals can be acquired. Even though no correlation was observed between hair EFV levels and Wisepill® adherence, this study still demonstrates the feasibility of using hair analysis to monitor long-term adherence among South African patients. To further explore the potential of monitoring adherence using ARV levels in hair, larger studies need to be conducted in cohorts with poorer adherence rates and more occurrences of virological failures compared to what was observed in the current study.

In order for hair analysis to become established within current forensic and clinical toxicology practices in South Africa, South Africans need to be willing to provide samples of their hair for analysis. However, collecting hair samples can be challenging, especially from cultural groups which have certain beliefs and superstitions against providing hair samples [255]. African females often wear their hair in braids which makes collecting samples of hair for analysis either difficult or impossible, whilst males are often clean-shaven and have no head hair that can be collected, or their hair is very short. Even

though the current study demonstrates some feasibility towards collecting hair samples from individuals of the African population for drug analysis, there were difficulties related to the hair sampling process which impacted the study. Hair samples were collected from only 59% of the participants enrolled into the parent study, with the majority of hair samples being collected from the female participants (93%). Future studies involving hair analysis for drugs among different populations in South Africa need to ensure that both male and female participants are provided with the necessary information surrounding the collection and analysis of hair samples to facilitate their willingness to be involved in hair analysis studies.

References

1. UNAIDS AIDS info. http://aidsinfo.unaids.org. Accessed 8 Jun 2017
2. Desai M, Dikshit R, Iyer G (2012) Antiretroviral drugs: Critical issues and recent advances. Indian J Pharmacol 44:288–298. doi: 10.4103/0253-7613.96296
3. World Health Organization (2016) Consolidated Guidelines on the use of Antiretroviral Drugs for treating and preventing HIV infection. Geneva, Switzerland
4. Department of Health (2014) National consolidated guidelines for the prevention of mother-to-child transmission of HIV (PMTCT) and the management of HIV in children, adolescents and adults. Pretoria, South Africa
5. Smith PF, DiCenzo R, Morse GD (2001) Clinical Pharmacokinetics of Non-Nucleoside Reverse Transcriptase Inhibitors. Clin Pharmacokinet 40:893–905. doi: 10.2165/00003088-200140120-00002
6. Rakhmanina NY, van den Anker JN (2010) Efavirenz in the therapy of HIV infection. Expert Opin Drug Metab Toxicol 6:95–103. doi: 10.1517/17425250903483207
7. Vrouenraets SM, Wit FW, Van Tongeren J, Lange JM (2007) Efavirenz: a review. Expert Opin Pharmacother 8:851–871. doi: 10.1517/14656566.8.6.851
8. Marzolini C, Telenti A, Decosterd L, Biollaz J, Buclin T (2001) Efavirenz plasma levels can predict treatment failure and central nervous system side effects in HIV-1-infected patients. AIDS 15:1193–1194. doi: 10.1097/00002030-200106150-00023
9. Ståhle L, Moberg L, Svensson J-O, Sönnerborg A (2004) Efavirenz plasma concentrations in HIV-infected patients: inter- and intraindividual variability and clinical effects. Ther Drug Monit 26:267–270.
10. Ward BA, Gorski J, Jones D, Hall S, Flockhart D, Desta Z (2003) The Cytochrome P450 2B6 (CYP2B6) Is the Main Catalyst of Efavirenz Primary and Secondary Metabolism: Implication for HIV/AIDS Therapy and Utility of Efavirenz as a Substrate Marker of CYP2B6 Catalytic Activity. J Pharmacol Exp Ther 306:287–300. doi: 10.1124/jpet.103.049601
11. Naidoo P, Chetty V V, Chetty M (2014) Impact of CYP polymorphisms, ethnicity and sex differences in metabolism on dosing strategies: the case of efavirenz. Eur J Clin Pharmacol 70:379–389. doi: 10.1007/s00228-013-1634-1
12. Rodriguez-Novoa S, Barreiro P, Rendon A, Jimenez-Nacher I, Gonzalez-Lahoz J, Soriano V (2005) Influence of 516G>T Polymorphisms at the Gene Encoding the CYP450-2B6 Isoenzyme on Efavirenz Plasma Concentrations in HIV-Infected Subjects. Clin Infect Dis 40:1358–1361. doi: 10.1086/429327
13. Haas DW, Ribaudo HJ, Kim RB, Tierney C, Wilkinson GR, Gulick RM, Clifford DB, Hulgan T, Marzolini C, Acosta EP (2004) Pharmacogenetics of efavirenz and central nervous system side effects: an Adult AIDS Clinical Trials Group study. AIDS 18:2391–2400. doi: 00002030-200412030-00006
14. Rotger M, Colombo S, Furrer H, Bleiber G, Buclin T, Lee BL, Keiser O, Biollaz J, Décosterd L, Telenti A (2005) Influence of CYP2B6 polymorphism on plasma and intracellular concentrations and toxicity of efavirenz and nevirapine in HIV-infected patients. Pharmacogenet Genomics 15:1–5. doi: 10.1097/01213011-200501000-00001
15. Chesney MA, Morin M, Sherr L (2000) Adherence to HIV combination therapy. Soc Sci Med 50:1599–1605. doi: 10.1016/S0277-9536(99)00468-2
16. Bartlett JG (2002) Addressing the Challenges of Adherence. J Acquir Immune Defic Syndr 29:S2–S10.
17. Ortego C, Huedo-Medina TB, Llorca J, Sevilla L, Santos P, Rodríguez E, Warren MR, Vejo J (2011) Adherence to Highly Active Antiretroviral Therapy (HAART): A Meta-Analysis. AIDS Behav 15:1381–1396. doi: 10.1007/s10461-011-9942-x

18. Gill CJ, Hamer DH, Simon JL, Thea DM, Sabin LL (2005) No room for complacency about adherence to antiretroviral therapy in sub-Saharan Africa. AIDS 19:1243–1249. doi: 10.1097/01.aids.0000180094.04652.3b
19. Berg KM, Arnsten JH (2006) Practical and Conceptual Challenges in Measuring Antiretroviral Adherence. J Acquir Immune Defic Syndr 43:S79–S87. doi: 10.1097/01.qai.0000248337.97814.66
20. Marcellin F, Spire B, Carrieri MP, Roux P (2013) Assessing adherence to antiretroviral therapy in randomized HIV clinical trials: a review of currently used methods. Expert Rev Anti Infect Ther 11:239–250. doi: 10.1586/eri.13.8
21. Osterberg L, Blaschke T (2005) Adherence to Medication. N Engl J Med 353:487–497. doi: 10.1056/NEJMra050100
22. Duong M, Piroth L, Peytavin G, Forte F, Kohli E, Grappin M, Buisson M, Chavanet P, Portier H (2001) Value of Patient Self-Report and Plasma Human Immunodeficiency Virus Protease Inhibitor Level as Markers of Adherence to Antiretroviral Therapy: Relationship to Virologic Response. Clin Infect Dis 33:386–392. doi: 10.1086/321876
23. Back D, Gibbons S, Khoo S (2006) An Update on Therapeutic Drug Monitoring for Antiretroviral Drugs. Ther Drug Monit 28:468–473. doi: 10.1097/01.ftd.0000211825.57984.41
24. Back D, Gatti G, Fletcher C, Garaffo R, Haubrich R, Hoetelmans R, Kurowski M, Luber A, Merry C, Perno C (2002) Therapeutic drug monitoring in HIV infection: current status and future directions. AIDS 16:S5–S37.
25. Nettles RE, Kieffer TL, Parsons T, Johnson J, Cofrancesco J, Gallant JE, Carson KA, Siliciano RF, Flexner C (2006) Marked Intraindividual Variability in Antiretroviral Concentrations May Limit the Utility of Therapeutic Drug Monitoring. Clin Infect Dis 42:1189–1196. doi: 10.1086/501458
26. Podsadecki TJ, Vrijens BC, Tousset EP, Rode RA, Hanna GJ (2008) “White Coat Compliance” Limits the Reliability of Therapeutic Drug Monitoring in HIV-1—Infected Patients. HIV Clin Trials 9:238–246. doi: 10.1310/hct0904-238
27. Gandhi M, Greenblatt RM (2002) Hair It Is: The Long and Short of Monitoring Antiretroviral Treatment. Ann Intern Med 137:696–697. doi: 10.7326/0003-4819-137-8-200210150-00016
28. García PR, Côté JK (2003) Factors Affecting Adherence to Antiretroviral Therapy in People Living with HIV/AIDS. J Assoc Nurses AIDS care 14:37–45. doi: 10.1177/1055329003252424
29. Bhat VG, Ramburuth M, Singh M, Titi O, Antony AP, Chiya L, Irusen EM, Mtyapi PP, Mofoka ME, Zibeke A, Chere-Sao LA, Gwadiso N, Sethathi NC, Mbondwana SR, Msengana M (2010) Factors associated with poor adherence to anti-retroviral therapy in patients attending a rural health centre in South Africa. Eur J Clin Microbiol Infect Dis 29:947–953. doi: 10.1007/s10096-010-0949-4
30. Ammassari A, Trotta MP, Murri R, Castelli F, Narciso P, Noto P, Vecchiet J, Monforte AD, Wu AW, Antinori A (2002) Correlates and Predictors of Adherence to Highly Active Antiretroviral Therapy: Overview of Published Literature. J Acquir Immune Defic Syndr 31:S123–S127. doi: 10.1097/00126334-200212153-00007
31. Chander G, Himelhoch S, Moore RD (2006) Substance abuse and psychiatric disorders in HIV-positive patients: Epidemiology and impact on antiretroviral therapy. Drugs 66:769–789. doi: 10.2165/00003495-200666060-00004
32. Mellins CA, Havens J, McDonnell C, Lichtenstein C, Uldall K, Chesney M, Santamaria EK, Bell J (2009) Adherence to antiretroviral medications and medical care in HIV-infected adults diagnosed with mental and substance abuse disorders. AIDS Care 21:168–177. doi: 10.1080/09540120802001705
33. Azar MM, Springer SA, Meyer JP, Altice FL (2010) A systematic review of the impact of

alcohol use disorders on HIV treatment outcomes, adherence to antiretroviral therapy and health care utilization. Drug Alcohol Depend 112:178–193. doi: 10.1016/j.drugalcdep.2010.06.014

34. Hendershot CS, Stoner SA, Pantalone DW, Simoni JM (2009) Alcohol Use and Antiretroviral Adherence: Review and Meta-Analysis. J Acquir Immune Defic Syndr 52:180–202. doi: 10.1097/QAI.0b013e3181b18b6e
35. Shuper PA, Neuman M, Kanteres F, Baliunas D, Joharchi N, Rehm J (2010) Causal Considerations on Alcohol and HIV/AIDS - A Systematic Review. Alcohol Alcohol 45:1–8. doi: 10.1093/alcalc/agp091
36. Schneider M, Neuman M, Chersich M, Parry C (2012) Alcohol and Antiretroviral Therapy - A Lethal Cocktail. J AIDS Clin Res 1:1–8. doi: 10.4172/2155-6113.S1-005
37. Pandrea I, Happel K, Amedee A, Bagby G, Nelson S (2010) Alcohol 's Role in HIV Transmission and Disease Progression. Alcohol Res Health 33:203–218.
38. Hahn JA, Woolf-King SE, Muyindike W (2011) Adding fuel to the fire: Alcohol's effect on the HIV epidemic in sub-saharan africa. Curr HIV/AIDS Rep 8:172–180. doi: 10.1007/s11904-011-0088-2
39. World Health Organization (2014) Global status report on alcohol and health 2014. Geneva, Switzerland
40. Kader R, Seedat S, Govender R, Koch JR, Parry CD (2014) Hazardous and Harmful use of Alcohol and/or Other Drugs and Health Status Among South African Patients Attending HIV Clinics. AIDS Behav 18:525–534. doi: 10.1007/s10461-013-0587-9
41. Kader R, Govender R, Seedat S, Koch JR, Parry C (2015) Understanding the Impact of Hazardous and Harmful Use of Alcohol and/or Other Drugs on ARV Adherence and Disease Progression. PLoS One 10:e0125088. doi: 10.1371/journal.pone.0125088
42. Nakimuli-Mpungu E, Bass JK, Alexandre P, Mills EJ, Musisi S, Ram M, Katabira E, Nachega JB (2012) Depression, alcohol use and adherence to antiretroviral therapy in sub-Saharan Africa: A systematic review. AIDS Behav 16:2101–2108. doi: 10.1007/s10461-011-0087-8
43. Morojele NK, Kekwaletswe CT, Nkosi S (2014) Associations Between Alcohol Use, Other Psychosocial Factors, Structural Factors and Antiretroviral Therapy (ART) Adherence Among South African ART Recipients. AIDS Behav 18:519–524. doi: 10.1007/s10461-013-0583-0
44. Magidson JF, Saal W, Nel A, Remmert JE, Kagee A (2016) Relationship between depressive symptoms, alcohol use, and antiretroviral therapy adherence among HIV-infected, clinic-attending patients in South Africa. J Health Psychol 1–8. doi: 10.1177/1359105316628743
45. Cerutti B, Broers B, Masetsibi M, Faturiyele O, Toti-Mokoteli L, Motlatsi M, Bader J, Klimkait T, Labhardt ND (2016) Alcohol use and depression: link with adherence and viral suppression in adult patients on antiretroviral therapy in rural Lesotho, Southern Africa: a cross-sectional study. BMC Public Health 16:947–953. doi: 10.1186/s12889-016-3209-4
46. Sileo KM, Simbayi LC, Abrams A, Cloete A, Kiene SM (2016) The role of alcohol use in antiretroviral adherence among individuals living with HIV in South Africa: Event-level findings from a daily diary study. Drug Alcohol Depend 167:103–111. doi: 10.1016/j.drugalcdep.2016.07.028
47. Kekwaletswe C, Morojele N (2014) Alcohol use, antiretroviral therapy adherence, and preferences regarding an alcohol-focused adherence intervention in patients with human immunodeficiency virus. Patient Prefer Adherence 8:401–413. doi: 10.2147/PPA.S55547
48. Kekwaletswe CT, Morojele NK (2014) Patterns and predictors of antiretroviral therapy use among alcohol drinkers at HIV clinics in Tshwane, South Africa. AIDS Care 26:S78–

S82. doi: 10.1080/09540121.2014.906558
49. Myers B, Sorsdahl K, Morojele NK, Kekwaletswe C, Shuper PA, Parry CDH (2017) “In this thing I have everything I need”: perceived acceptability of a brief alcohol-focused intervention for people living with HIV. AIDS Care 29:209–213. doi: 10.1080/09540121.2016.1211242
50. Wechsberg WM, Jewkes R, Novak SP, Kline T, Myers B, Browne FA, Carney T, Morgan Lopez AA, Parry C (2013) A brief intervention for drug use, sexual risk behaviours and violence prevention with vulnerable women in South Africa: a randomised trial of the Women’s Health CoOp. BMJ Open 3:1–10. doi: 10.1136/bmjopen-2013-002622
51. Zule W, Myers B, Carney T, Novak SP, McCormick K, Wechsberg WM (2014) Alcohol and drug use outcomes among vulnerable women living with HIV: results from the Western Cape Women’s Health CoOp. AIDS Care 26:1494–1499. doi: 10.1080/09540121.2014.933769
52. Saunders JB, Aasland OG, Babor TF, de la Fuente JR, Grant M (1993) Development of the Alcohol Use Disorders Identification Test (AUDIT): WHO Collaborative Project on Early Detection of Persons with Harmful Alcohol Consumption--II. Addiction 88:791–804. doi: 10.1111/j.1360-0443.1993.tb02093.x
53. Berman AH, Bergman H, Palmstierna T, Schlyter F (2003) DUDIT Manual: The Drug Use Disorders Identification Test. Stockholm: Karolinska Institutet, Department of Clinical Neuroscience
54. Baumgartner AM, Jones PF, Baumgartner W, Black CT (1979) Radioimmunoassy of hair for determining opiate-abuse histories. J Nucl Med 748–752.
55. Cooper GAA (2011) Hair testing is taking root. Ann Clin Biochem 48:516–530. doi: 10.1258/acb.2011.011112
56. Cooper GAA, Kronstrand R, Kintz P (2012) Society of Hair Testing guidelines for drug testing in hair. Forensic Sci Int 218:20–24. doi: 10.1016/j.forsciint.2011.10.024
57. Harkey MR (1993) Anatomy and physiology of hair. Forensic Sci Int 63:9–18. doi: 10.1016/0379-0738(93)90255-9
58. Montagna W, Van Scott E (1958) The anatomy of the hair follicle. In: Montagna W, Ellis R (eds) The Biology of Hair Growth. Academic Press, New York, pp 39–64
59. Ryder M (1958) Nutritional factors influencing hair and wool growth. In: Montagna W, Ellis R (eds) The Biology of Hair Growth. Academic Press, New York, pp 305–334
60. Henderson GL (1993) Mechanisms of drug incorporation into hair. Forensic Sci Int 63:19–29. doi: 10.1016/0379-0738(93)90256-A
61. Kronstrand R, Scott K (2007) Drug Incorporation into Hair. In: Kintz P (ed) Analytical and Practical Aspects of Drug Testing in Hair. CRC Press, Boca Raton, pp 1–23
62. Pragst F, Balikova MA (2006) State of the art in hair analysis for detection of drug and alcohol abuse. Clin Chim Acta 370:17–49. doi: 10.1016/j.cca.2006.02.019
63. Cooper GAA (2015) Anatomy and Physiology of Hair, and Principles for its Collection. In: Kintz P, Salomone A, Vincenti M (eds) Hair Analysis in Clinical and Forensic Toxicology. Academic Press, New York, pp 1–17
64. Borges CR, Roberts JC, Wilkins DG, Rollins DE (2003) Cocaine, Benzoylecgonine Amphetamine, and N-Acetylamphetamine Binding to Melanin Subtypes. J Anal Toxicol 27:125–134. doi: 10.1093/jat/27.3.125
65. Borges CR, Wilkins DG, Rollins DE (2001) Amphetamine and N-Acetylamphetamine Incorporation into Hair: An Investigation of the Potential Role of Drug Basicity in Hair Color Bias. J Anal Toxicol 25:221–227. doi: 10.1093/jat/25.4.221
66. Rollins DE, Wilkins DG, Krueger GG, Augsburger MP, Mizuno A, O’Neal C, Borges CR, Slawson MH (2003) The Effect of Hair Color on the Incorporation of Codeine into Human Hair. J Anal Toxicol 27:545–551. doi: 10.1093/jat/27.8.545
67. Slawson MH, Wilkins DG, Rollins DE (1998) The Incorporation of Drugs into Hair:

Relationship of Hair Color and Melanin Concentration to Phencyclidine Incorporation. J Anal Toxicol 22:406–413. doi: 10.1093/jat/22.6.406

68. Eser HP, Pötsch L, Skopp G, Moeller MR (1997) Influence of sample preparation on analytical results: Drug analysis [GC/MS] on hair snippets versus hair powder using various extraction methods. Forensic Sci Int 84:271–279. doi: 10.1016/S0379-0738(96)02071-3
69. Chiarotti M (1993) Overview on extraction procedures. Forensic Sci Int 63:161–170. doi: 10.1016/0379-0738(93)90270-K
70. Niemelä O (2007) Biomarkers in alcoholism. Clin Chim Acta 377:39–49. doi: 10.1016/j.cca.2006.08.035
71. Conigrave KM, Degenhardt LJ, Whitfield JB, Saunders JB, Helander A, Tabakoff B (2002) CDT, GGT, and AST As Markers of Alcohol Use: The WHO/ISBRA Collaborative Project. Alcohol Clin Exp Res 26:332–339. doi: 10.1111/j.1530-0277.2002.tb02542.x
72. Maenhout TM, De Buyzere ML, Delanghe JR (2013) Non-oxidative ethanol metabolites as a measure of alcohol intake. Clin Chim Acta 415:322–329. doi: 10.1016/j.cca.2012.11.014
73. Wurst FM, Thon N, Yegles M, Schrück A, Preuss UW, Weinmann W (2015) Ethanol Metabolites: Their Role in the Assessment of Alcohol Intake. Alcohol Clin Exp Res 39:2060–2072. doi: 10.1111/acer.12851
74. Crunelle CL, Yegles M, Nuijs ALN van, Covaci A, De Doncker M, Maudens KE, Sabbe B, Dom G, Lambert WE, Michielsen P, Neels H (2014) Hair ethyl glucuronide levels as a marker for alcohol use and abuse: A review of the current state of the art. Drug Alcohol Depend 134:1–11. doi: 10.1016/j.drugalcdep.2013.10.008
75. Kintz P (2015) 2014 Consensus for the use of alcohol markers in hair for assessment of both abstinence and chronic excessive alcohol consumption. Forensic Sci Int 249:A1–A2. doi: 10.1016/j.forsciint.2014.11.001
76. Society of Hair Testing 2016 Consensus for the Use of Alcohol Markers in Hair for the Assessment of both Abstinence and Chronic Excessive Alcohol Consumption. http://www.soht.org/images/pdf/Revision 2016_Alcoholmarkers.pdf. Accessed 26 Jan 2017
77. Hartwig S, Auwärter V, Pragst F (2003) Effect of hair care and hair cosmetics on the concentrations of fatty acid ethyl esters in hair as markers of chronically elevated alcohol consumption. Forensic Sci Int 131:90–97. doi: 10.1016/S0379-0738(02)00412-7
78. Suesse S, Pragst F, Mieczkowski T, Selavka CM, Elian A, Sachs H, Hastedt M, Rothe M, Campbell J (2012) Practical experiences in application of hair fatty acid ethyl esters and ethyl glucuronide for detection of chronic alcohol abuse in forensic cases. Forensic Sci Int 218:82–91. doi: 10.1016/j.forsciint.2011.10.006
79. Cabarcos P, Álvarez I, Tabernero MJ, Bermejo AM (2015) Determination of direct alcohol markers: a review. Anal Bioanal Chem 407:4907–4925. doi: 10.1007/s00216-015-8701-7
80. Pragst F, Yegles M (2008) Determination of Fatty Acid Ethyl Esters (FAEE) and Ethyl Glucuronide (EtG) in Hair: A Promising Way for Retrospective Detection of Alcohol Abuse During Pregnancy? Ther Drug Monit 30:255–263. doi: 10.1097/FTD.0b013e318167d602
81. Pragst F, Rothe M, Moench B, Hastedt M, Herre S, Simmert D (2010) Combined use of fatty acid ethyl esters and ethyl glucuronide in hair for diagnosis of alcohol abuse: Interpretation and advantages. Forensic Sci Int 196:101–110. doi: 10.1016/j.forsciint.2009.12.028
82. Foti RS, Fisher MB (2005) Assessment of UDP-glucuronosyltransferase catalyzed formation of ethyl glucuronide in human liver microsomes and recombinant UGTs. Forensic Sci Int 153:109–116. doi: 10.1016/j.forsciint.2004.12.003
83. Goll M, Schmitt G, Ganssmann B, Aderjan RE (2002) Excretion Profiles of Ethyl Glucuronide in Human Urine after Internal Dilution. J Anal Toxicol 26:262–266. doi: 10.1093/jat/26.5.262

84. Dahl H, Stephanson N, Beck O, Helander A (2002) Comparison of Urinary Excretion Characteristics of Ethanol and Ethyl Glucuronide. J Anal Toxicol 26:201–204. doi: 10.1093/jat/26.4.201
85. Halter CC, Dresen S, Auwaerter V, Wurst FM, Weinmann W (2008) Kinetics in serum and urinary excretion of ethyl sulfate and ethyl glucuronide after medium dose ethanol intake. Int J Legal Med 122:123–128. doi: 10.1007/s00414-007-0180-8
86. Schmitt G, Aderjan R, Keller T, Wu M (1995) Ethyl Glucuronide: An Unusual Ethanol Metabolite in Humans. Synthesis, Analytical Data, and Determination in Serum and Urine. J Anal Toxicol 19:91–94. doi: 10.1093/jat/19.2.91
87. Wurst F (1999) Ethyl glucuronide - a marker of alcohol consumption and a relapse marker with clinical and forensic implications. Alcohol Alcohol 34:71–77. doi: 10.1093/alcalc/34.1.71
88. Kharbouche H, Steiner N, Morelato M, Staub C, Boutrel B, Mangin P, Sporkert F, Augsburger M (2010) Influence of ethanol dose and pigmentation on the incorporation of ethyl glucuronide into rat hair. Alcohol 44:507–514. doi: 10.1016/j.alcohol.2010.05.001
89. Schräder J, Rothe M, Pragst F (2012) Ethyl glucuronide concentrations in beard hair after a single alcohol dose: evidence for incorporation in hair root. Int J Legal Med 126:791–799. doi: 10.1007/s00414-012-0729-z
90. Nakahara Y, Takahashi K, Kikura R (1995) Hair Analysis for Drugs of Abuse. X. Effect of Physicochemical Properties of Drugs on the Incorporation Rates into Hair. Biol Pharm Bull 18:1223–1227. doi: 10.1248/bpb.18.1223
91. Skopp G, Schmitt G, Potsch L, Dronner P, Aderjan R, Mattern R (2000) Ethyl Glucuronide in Human Hair. Alcohol Alcohol 35:283–285. doi: 10.1093/alcalc/35.3.283
92. Alt A, Janda I, Seidl S, Wurst F (2000) Determination of ethyl glucuronide in hair samples. Alcohol Alcohol 35:313–314. doi: 10.1093/alcalc/35.3.313
93. Janda I, Weinmann W, Kuehnle T, Lahode M, Alt A (2002) Determination of ethyl glucuronide in human hair by SPE and LC–MS/MS. Forensic Sci Int 128:59–65. doi: 10.1016/S0379-0738(02)00163-9
94. Pragst F (2015) Alcohol Biomarkers in Hair. In: Kintz P, Salomone A, Vincenti M (eds) Hair Analysis in Clinical and Forensic Toxicology. Academic Press, New York, pp 71–139
95. Albermann ME, Musshoff F, Madea B (2010) A fully validated high-performance liquid chromatography-tandem mass spectrometry method for the determination of ethyl glucuronide in hair for the proof of strict alcohol abstinence. Anal Bioanal Chem 396:2441–2447. doi: 10.1007/s00216-009-3388-2
96. Concheiro M, Cruz A, Mon M, de Castro A, Quintela O, Lorenzo A, Lopez-Rivadulla M (2009) Ethylglucuronide Determination in Urine and Hair from Alcohol Withdrawal Patients. J Anal Toxicol 33:155–161. doi: 10.1093/jat/33.3.155
97. Kronstrand R, Brinkhagen L, Nyström FH (2012) Ethyl glucuronide in human hair after daily consumption of 16 or 32g of ethanol for 3 months. Forensic Sci Int 215:51–55. doi: 10.1016/j.forsciint.2011.01.044
98. Pirro V, Di Corcia D, Pellegrino S, Vincenti M, Sciutteri B, Salomone A (2011) A study of distribution of ethyl glucuronide in different keratin matrices. Forensic Sci Int 210:271–277. doi: 10.1016/j.forsciint.2011.03.026
99. Morini L, Politi L, Groppi A, Stramesi C, Polettini A (2006) Determination of ethyl glucuronide in hair samples by liquid chromatography/electrospray tandem mass spectrometry. J Mass Spectrom 41:34–42. doi: 10.1002/jms.943
100. Imbert L, Gaulier JM, Dulaurent S, Morichon J, Bevalot F, Izac P, Lachâtre G (2014) Improved liquid chromatography-tandem mass spectrometric method for the determination of ethyl glucuronide concentrations in hair: Applications to forensic cases. Int J Legal Med 128:53–58. doi: 10.1007/s00414-013-0894-8

101. Kerekes I, Yegles M, Grimm U, Wennig R (2008) Ethyl Glucuronide Determination: Head Hair versus Non-Head Hair. Alcohol Alcohol 44:62–66. doi: 10.1093/alcalc/agn096
102. Kharbouche H, Sporkert F, Troxler S, Augsburger M, Mangin P, Staub C (2009) Development and validation of a gas chromatography–negative chemical ionization tandem mass spectrometry method for the determination of ethyl glucuronide in hair and its application to forensic toxicology. J Chromatogr B 877:2337–2343. doi: 10.1016/j.jchromb.2008.11.046
103. Lamoureux F, Gaulier JM, Sauvage FL, Mercerolle M, Vallejo C, Lachâtre G (2009) Determination of ethyl-glucuronide in hair for heavy drinking detection using liquid chromatography-tandem mass spectrometry following solid-phase extraction. Anal Bioanal Chem 394:1895–1901. doi: 10.1007/s00216-009-2863-0
104. Martins Ferreira L, Binz T, Yegles M (2012) The influence of ethanol containing cosmetics on ethyl glucuronide concentration in hair. Forensic Sci Int 218:123–125. doi: 10.1016/j.forsciint.2011.10.015
105. Albermann ME, Musshoff F, Aengenheister L, Madea B (2012) Investigations on the influence of different grinding procedures on measured ethyl glucuronide concentrations in hair determined with an optimized and validated LC-MS/MS method. Anal Bioanal Chem 403:769–776. doi: 10.1007/s00216-012-5926-6
106. Mönch B, Becker R, Nehls I (2013) Quantification of ethyl glucuronide in hair: Effect of milling on extraction efficiency. Alcohol Alcohol 48:558–563. doi: 10.1093/alcalc/agt059
107. Kummer N, Wille SMR, Di Fazio V, Ramirez Fernandez MDM, Yegles M, Lambert WEE, Samyn N (2014) Impact of the Grinding Process on the Quantification of Ethyl Glucuronide in Hair Using a Validated UPLC-ESI-MS-MS Method. J Anal Toxicol 39:17–23. doi: 10.1093/jat/bku108
108. Mönch B, Becker R, Nehls I (2014) Determination of ethyl glucuronide in hair: a rapid sample pretreatment involving simultaneous milling and extraction. Int J Legal Med 128:69–72. doi: 10.1007/s00414-013-0939-z
109. Jurado C, Soriano T, Giménez MP, Menéndez M (2004) Diagnosis of chronic alcohol consumption: Hair analysis for ethyl-glucuronide. Forensic Sci Int 145:161–166. doi: 10.1016/j.forsciint.2004.04.031
110. Pirro V, Di Corcia D, Seganti F, Salomone A, Vincenti M (2013) Determination of ethyl glucuronide levels in hair for the assessment of alcohol abstinence. Forensic Sci Int 232:229–236. doi: 10.1016/j.forsciint.2013.07.024
111. Kintz P, Villain M, Vallet E, Etter M, Salquebre G, Cirimele V (2008) Ethyl glucuronide: Unusual distribution between head hair and pubic hair. Forensic Sci Int 176:87–90. doi: 10.1016/j.forsciint.2007.08.012
112. Tarcomnicu I, van Nuijs ALN, Aerts K, De Doncker M, Covaci A, Neels H (2010) Ethyl glucuronide determination in meconium and hair by hydrophilic interaction liquid chromatography–tandem mass spectrometry. Forensic Sci Int 196:121–127. doi: 10.1016/j.forsciint.2009.12.043
113. Yaldiz F, Daglioglu N, Hilal A, Keten A, Gülmen MK (2013) Determination of ethyl glucuronide in human hair by hydrophilic interaction liquid chromatography–tandem mass spectrometry. J Forensic Leg Med 20:799–802. doi: 10.1016/j.jflm.2013.06.013
114. Oppolzer D, Barroso M, Passarinha L, Gallardo E (2016) Determination of ethyl glucuronide and fatty acid ethyl esters in hair samples. Biomed Chromatogr 31:1–12. doi: 10.1002/bmc.3858
115. Appenzeller BMR, Schuman M, Yegles M, Wennig R (2007) Ethyl glucuronide concentration in hair is not influenced by pigmentation. Alcohol Alcohol 42:326–327. doi: 10.1093/alcalc/agm016
116. Morini L, Zucchella A, Polettini A, Politi L, Groppi A (2010) Effect of bleaching on ethyl glucuronide in hair: An in vitro experiment. Forensic Sci Int 198:23–27. doi:

10.1016/j.forsciint.2009.11.005

117. Kerekes I, Yegles M (2013) Coloring, Bleaching, and Perming: Influence on EtG Content in Hair. Ther Drug Monit 35:527–529. doi: 10.1097/FTD.0b013e31828ca246
118. Oppolzer D, Barroso M, Gallardo E (2016) Determination of ethyl glucuronide in hair to assess excessive alcohol consumption in a student population. Anal Bioanal Chem 408:2027–2034. doi: 10.1007/s00216-015-9155-7
119. Crunelle CL, Yegles M, De Doncker M, Dom G, Cappelle D, Maudens KE, van Nuijs ALN, Covaci A, Neels H (2015) Influence of repeated permanent coloring and bleaching on ethyl glucuronide concentrations in hair from alcohol-dependent patients. Forensic Sci Int 247:18–22. doi: 10.1016/j.forsciint.2014.11.023
120. Ettlinger J, Yegles M (2016) Influence of thermal hair straightening on cannabis and cocaine content in hair. Forensic Sci Int 265:13–16. doi: 10.1016/j.forsciint.2016.01.002
121. Morini L, Politi L, Polettini A (2009) Ethyl glucuronide in hair. A sensitive and specific marker of chronic heavy drinking. Addiction 104:915–920. doi: 10.1111/j.1360-0443.2009.02535.x
122. Crunelle CL, Cappelle D, Covaci A, van Nuijs ALN, Maudens KE, Sabbe B, Dom G, Michielsen P, Yegles M, Neels H (2014) Hair ethyl glucuronide as a biomarker of alcohol consumption in alcohol-dependent patients: Role of gender differences. Drug Alcohol Depend 141:163–166. doi: 10.1016/j.drugalcdep.2014.05.014
123. Gareri J, Rao C, Koren G (2014) Examination of sex differences in fatty acid ethyl ester and ethyl glucuronide hair analysis. Drug Test Anal 6:30–36. doi: 10.1002/dta.1653
124. Crunelle CL, Neels H, Maudens K, De Doncker M, Cappelle D, Matthys F, Dom G, Fransen E, Michielsen P, De Keukeleire S, Covaci A, Yegles M (2017) Influence of Body Mass Index on Hair Ethyl Glucuronide Concentrations. Alcohol Alcohol 52:19–23. doi: 10.1093/alcalc/agw079
125. Pianta A, Liniger B, Baumgartner MR (2013) Ethyl glucuronide in scalp and non-head hair: An intra-individual comparison. Alcohol Alcohol 48:295–302. doi: 10.1093/alcalc/agt012
126. L. Crunelle C, Cappelle D, Yegles M, De Doncker M, Michielsen P, Dom G, Van Nuijs ALN, Maudens KE, Covaci A, Neels H (2016) Ethyl glucuronide concentrations in hair: A controlled alcohol-dosing study in healthy volunteers. Anal Bioanal Chem 408:2019–2025. doi: 10.1007/s00216-015-9117-0
127. Appenzeller BMR, Agirman R, Neuberg P, Yegles M, Wennig R (2007) Segmental determination of ethyl glucuronide in hair: A pilot study. Forensic Sci Int 173:87–92. doi: 10.1016/j.forsciint.2007.01.025
128. Høiseth G, Morini L, Polettini A, Christophersen A, Mørland J (2009) Ethyl Glucuronide in Hair Compared With Traditional Alcohol Biomarkers-A Pilot Study of Heavy Drinkers Referred to an Alcohol Detoxification Unit. Alcohol Clin Exp Res 33:812–816. doi: 10.1111/j.1530-0277.2009.00900.x
129. Politi L, Morini L, Leone F, Polettini A (2006) Ethyl glucuronide in hair: Is it a reliable marker of chronic high levels of alcohol consumption? Addiction 101:1408–12. doi: 10.1111/j.1360-0443.2006.01537.x
130. Farré M, de la Torre R, Llorente M, Lamas X, Ugena B, Segura J, Camí J (1993) Alcohol and cocaine interactions in humans. J Pharmacol Exp Ther 266:1364–1373.
131. Farré M, de la Torre R, González ML, Terán MT, Roset PN, Menoyo E, Camí J (1997) Cocaine and alcohol interactions in humans: neuroendocrine effects and cocaethylene metabolism. J Pharmacol Exp Ther 283:164–176.
132. Harris DS, Everhart ET, Mendelson J, Jones RT (2003) The pharmacology of cocaethylene in humans following cocaine and ethanol administration. Drug Alcohol Depend 72:169–182. doi: 10.1016/S0376-8716(03)00200-X
133. Moore C, Coulter C, Crompton K (2007) Determination of cocaine, benzoylecgonine, cocaethylene and norcocaine in human hair using solid-phase extraction and liquid

chromatography with tandem mass spectrometric detection. J Chromatogr B 859:208–212. doi: 10.1016/j.jchromb.2007.09.037

134. Pego AMF, Roveri FL, Kuninari RY, Leyton V, Miziara ID, Yonamine M (2017) Determination of cocaine and its derivatives in hair samples by liquid phase microextraction (LPME) and gas chromatography–mass spectrometry (GC–MS). Forensic Sci Int 274:83–90. doi: 10.1016/j.forsciint.2016.12.024
135. López-Guarnido O, Álvarez I, Gil F, Rodrigo L, Cataño HC, Bermejo AM, Tabernero MJ, Pla A, Hernández AF (2013) Hair testing for cocaine and metabolites by GC/MS: criteria to quantitatively assess cocaine use. J Appl Toxicol 33:838–844. doi: 10.1002/jat.2741
136. Natekar A, Matok I, Walasek P, Rao C, Clare-Fasullo G, Koren G (2012) Cocaethylene as a hair biomarker to predict heavy alcohol exposure among cocaine users. J Popul Ther Clin Pharmacol 19:e466–e472.
137. Pragst F, Spiegel K, Sporkert F, Bohnenkamp M (2000) Are there possibilities for the detection of chronically elevated alcohol consumption by hair analysis? A report about the state of investigation. Forensic Sci Int 107:201–223. doi: 10.1016/S0379-0738(99)00164-4
138. Musshoff F (2002) Chromatographic methods for the determination of markers of chronic and acute alcohol consumption. J Chromatogr B 781:457–480. doi: 10.1016/S1570-0232(02)00691-8
139. Politi L, Zucchella A, Morini L, Stramesi C, Polettini A (2007) Markers of chronic alcohol use in hair: Comparison of ethyl glucuronide and cocaethylene in cocaine users. Forensic Sci Int 172:23–27. doi: 10.1016/j.forsciint.2006.11.007
140. Vogliardi S, Tucci M, Stocchero G, Ferrara SD, Favretto D (2015) Sample preparation methods for determination of drugs of abuse in hair samples: A review. Anal Chim Acta 857:1–27. doi: 10.1016/j.aca.2014.06.053
141. Baciu T, Borrull F, Aguilar C, Calull M (2015) Recent trends in analytical methods and separation techniques for drugs of abuse in hair. Anal Chim Acta 856:1–26. doi: 10.1016/j.aca.2014.06.051
142. Vincenti M, Salomone A, Gerace E, Pirro V (2013) Application of mass spectrometry to hair analysis for forensic toxicological investigations. Mass Spectrom Rev 32:312–332. doi: 10.1002/mas.21364
143. Wada M, Ikeda R, Kuroda N, Nakashima K (2010) Analytical methods for abused drugs in hair and their applications. Anal Bioanal Chem 397:1039–1067. doi: 10.1007/s00216-010-3569-z
144. Curtis J, Greenberg M (2008) Screening for drugs of abuse: Hair as an alternative matrix: a review for the medical toxicologist. Clin Toxicol 46:22–34. doi: 10.1080/15563650701261462
145. Favretto D, Vogliardi S, Stocchero G, Nalesso A, Tucci M, Ferrara SD (2011) High performance liquid chromatography–high resolution mass spectrometry and micropulverized extraction for the quantification of amphetamines, cocaine, opioids, benzodiazepines, antidepressants and hallucinogens in 2.5mg hair samples. J Chromatogr A 1218:6583–6595. doi: 10.1016/j.chroma.2011.07.050
146. Koster RA, Alffenaar J-WC, Greijdanus B, VanDerNagel JEL, Uges DRA (2014) Fast and Highly Selective LC-MS/MS Screening for THC and 16 Other Abused Drugs and Metabolites in Human Hair to Monitor Patients for Drug Abuse. Ther Drug Monit 36:234–243. doi: 10.1097/FTD.0b013e3182a377e8
147. Lendoiro E, Jiménez-Morigosa C, Cruz A, Páramo M, López-Rivadulla M, de Castro A (2017) An LC-MS/MS methodological approach to the analysis of hair for amphetamine-type-stimulant (ATS) drugs, including selected synthetic cathinones and piperazines. Drug Test Anal 9:96–105. doi: 10.1002/dta.1948
148. Montesano C, Johansen SS, Nielsen MKK (2014) Validation of a method for the targeted

analysis of 96 drugs in hair by UPLC–MS/MS. J Pharm Biomed Anal 88:295–306. doi: 10.1016/j.jpba.2013.08.050

149. Han E, Yang H, Seol I, Park Y, Lee B, Song JM (2013) Segmental hair analysis and estimation of methamphetamine use pattern. Int J Legal Med 127:405–411. doi: 10.1007/s00414-012-0766-7
150. Jakobsson G, Kronstrand R (2014) Segmental analysis of amphetamines in hair using a sensitive UHPLC-MS/MS method. Drug Test Anal 6:22–29. doi: 10.1002/dta.1637
151. Miyaguchi H, Kakuta M, Iwata YT, Matsuda H, Tazawa H, Kimura H, Inoue H (2007) Development of a micropulverized extraction method for rapid toxicological analysis of methamphetamine in hair. J Chromatogr A 1163:43–48. doi: 10.1016/j.chroma.2007.06.017
152. Paulsen RB, Wilkins DG, Slawson MH, Shaw K, Rollins DE (2001) Effect of four laboratory decontamination procedures on the quantitative determination of cocaine and metabolites in hair by HPLC-MS. J Anal Toxicol 25:490–496. doi: 10.1093/jat/25.7.490
153. Romano G, Barbera N, Lombardo I (2001) Hair testing for drugs of abuse: evaluation of external cocaine contamination and risk of false positives. Forensic Sci Int 123:119–129. doi: 10.1016/S0379-0738(01)00539-4
154. Schaffer MI, Wang W-L, Irving J (2002) An Evaluation of Two Wash Procedures for the Differentiation of External Contamination versus Ingestion in the Analysis of Human Hair Samples for Cocaine. J Anal Toxicol 26:485–488. doi: 10.1093/jat/26.7.485
155. Morris-Kukoski CL, Montgomery MA, Hammer RL (2014) Analysis of Extensively Washed Hair from Cocaine Users and Drug Chemists to Establish New Reporting Criteria. J Anal Toxicol 38:628–636. doi: 10.1093/jat/bku099
156. Gerace E, Veronesi A, Martra G, Salomone A, Vincenti M (2017) Study of cocaine incorporation in hair damaged by cosmetic treatments. Forensic Chem 3:69–73. doi: 10.1016/j.forc.2017.02.004
157. Kintz P, Mangin P (1995) Simultaneous determination of opiates, cocaine and major metabolites of cocaine in human hair by gas chromotography/mass spectrometry (GC/MS). Forensic Sci Int 73:93–100. doi: 10.1016/0379-0738(95)01725-X
158. Bucelli F, Fratini A, Bavazzano P, Comodo N (2009) Quantification of drugs of abuse and some stimulants in hair samples by liquid chromatography–electrospray ionization ion trap mass spectrometry. J Chromatogr B 877:3931–3936. doi: 10.1016/j.jchromb.2009.09.026
159. Imbert L, Dulaurent S, Mercerolle M, Morichon J, Lachâtre G, Gaulier J-M (2014) Development and validation of a single LC–MS/MS assay following SPE for simultaneous hair analysis of amphetamines, opiates, cocaine and metabolites. Forensic Sci Int 234:132–138. doi: 10.1016/j.forsciint.2013.11.004
160. Miller EI, Wylie FM, Oliver JS (2008) Simultaneous detection and quantification of amphetamines, diazepam and its metabolites, cocaine and its metabolites, and opiates in hair by LC-ESI-MS-MS using a single extraction method. J Anal Toxicol 32:457–469. doi: 10.1093/jat/32.7.457
161. Cordero R, Paterson S (2007) Simultaneous quantification of opiates, amphetamines, cocaine and metabolites and diazepam and metabolite in a single hair sample using GC–MS. J Chromatogr B 850:423–431. doi: 10.1016/j.jchromb.2006.12.021
162. Domínguez-Romero JC, García-Reyes JF, Molina-Díaz A (2011) Screening and quantitation of multiclass drugs of abuse and pharmaceuticals in hair by fast liquid chromatography electrospray time-of-flight mass spectrometry. J Chromatogr B 879:2034–2042. doi: 10.1016/j.jchromb.2011.05.034
163. Kronstrand R, Nyström I, Strandberg J, Druid H (2004) Screening for drugs of abuse in hair with ion spray LC–MS–MS. Forensic Sci Int 145:183–190. doi: 10.1016/j.forsciint.2004.04.034

164. Miyaguchi H, Inoue H (2011) Determination of amphetamine-type stimulants, cocaine and ketamine in human hair by liquid chromatography/linear ion trap–Orbitrap hybrid mass spectrometry. Analyst 136:3503–3511. doi: 10.1039/c0an00850h
165. Nielsen MKK, Johansen SS, Dalsgaard PW, Linnet K (2010) Simultaneous screening and quantification of 52 common pharmaceuticals and drugs of abuse in hair using UPLC–TOF-MS. Forensic Sci Int 196:85–92. doi: 10.1016/j.forsciint.2009.12.027
166. Odoardi S, Valentini V, De Giovanni N, Pascali VL, Strano-Rossi S (2017) High-throughput screening for drugs of abuse and pharmaceutical drugs in hair by liquid-chromatography-high resolution mass spectrometry (LC-HRMS). Microchem J 133:302–310. doi: 10.1016/j.microc.2017.03.050
167. Musshoff F, Madea B (2007) Analytical pitfalls in hair testing. Anal Bioanal Chem 388:1475–1494. doi: 10.1007/s00216-007-1288-x
168. Tsanaclis L, Wicks JFC (2008) Differentiation between drug use and environmental contamination when testing for drugs in hair. Forensic Sci Int 176:19–22. doi: 10.1016/j.forsciint.2007.08.009
169. Baumgartner WA, Hill VA (1993) Sample preparation techniques. Forensic Sci Int 63:121–135. doi: 10.1016/0379-0738(93)90266-D
170. Cairns T, Hill V, Schaffer M, Thistle W (2004) Removing and identifying drug contamination in the analysis of human hair. Forensic Sci Int 145:97–108. doi: 10.1016/j.forsciint.2004.04.024
171. Blank DL, Kidwell DA (1995) Decontamination procedures for drugs of abuse in hair: are they sufficient? Forensic Sci Int 70:13–38. doi: 10.1016/0379-0738(94)01617-E
172. Society of Hair Testing (1997) Society of hair testing. Forensic Sci Int 84:3–6. doi: 10.1016/S0379-0738(96)02042-7
173. Stout PR, Ropero-Miller JD, Baylor MR, Mitchell JM (2006) External contamination of hair with cocaine: evaluation of external cocaine contamination and development of performance-testing materials. J Anal Toxicol 30:490–500.
174. Kronstrand R, Andersson MC, Ahlner J, Larson G (2001) Incorporation of Selegiline Metabolites into Hair after Oral Selegiline Intake. J Anal Toxicol 25:594–601. doi: 10.1093/jat/25.7.594
175. Kronstrand R, Ahlner J, Dizdar N, Larson G (2003) Quantitative Analysis of Desmethylselegiline, Methamphetamine, and Amphetamine in Hair and Plasma from Parkinson Patients on Long-Term Selegiline Medication. J Anal Toxicol 27:135–141. doi: 10.1093/jat/27.3.135
176. Hubbard DL, Wilkins DG, Rollins DE (2000) The incorportation of cocaine and cocaine metabolites into hair: Effects of dose and pigmentation. Drug Metab Dispos 28:1464–1469.
177. Joseph RE, Su T-P, Cone EJ (1996) In Vitro Binding Studies of Drugs to Hair: Influence of Melanin and Lipids on Cocaine Binding to Caucasoid and Africoid Hair. J Anal Toxicol 20:338–344. doi: 10.1093/jat/20.6.338
178. Henderson GL, Harkey MR, Zhou C, Jones RT, Jacob P (1996) Incorporation of Isotopically Labeled Cocaine and Metabolites into Human Hair: 1. Dose-Response Relationships. J Anal Toxicol 20:1–12. doi: 10.1093/jat/20.1.1
179. Henderson GL, Harkey MR, Zhou C, Jones RT, Jacob P (1998) Incorporation of Isotopically Labeled Cocaine into Human Hair: Race as a Factor. J Anal Toxicol 22:156–165. doi: 10.1093/jat/22.2.156
180. Ropero-Miller JD, Huestis MA, Stout PR (2012) Cocaine Analytes in Human Hair: Evaluation of Concentration Ratios in Different Cocaine Sources, Drug-User Populations and Surface-Contaminated Specimens. J Anal Toxicol 36:390–398. doi: 10.1093/jat/bks050
181. Skopp G, Pötsch L, Moeller MR (1997) On cosmetically treated hair - aspects and pitfalls

of interpretation. Forensic Sci Int 84:43–52. doi: 10.1016/S0379-0738(96)02047-6
182. Jurado C, Kintz P, Menéndez M, Repetto M (1997) Influence of the cosmetic treatment of hair on drug testing. Int J Legal Med 110:159–163. doi: 10.1007/s004140050056
183. Takayama N, Tanaka S, Kizu R, Hayakawa K (1999) High-performance liquid chromatography study on effects of permanent wave, dye and decolorant treatments on methamphetamine and amphetamine in hair. Biomed Chromatogr 13:257–261. doi: 10.1002/(SICI)1099-0801(199906)13:4<257::AID-BMC830>3.0.CO;2-V
184. Hill V, Cairns T, Schaffer M (2008) Hair analysis for cocaine: Factors in laboratory contamination studies and their relevance to proficiency sample preparation and hair testing practices. Forensic Sci Int 176:23–33. doi: 10.1016/j.forsciint.2007.08.011
185. van Zyl GU, van Mens TE, McIlleron H, Zeier M, Nachega JB, Decloedt E, Malavazzi C, Smith P, Huang Y, Merwe L van der, Gandhi M, Maartens G (2011) Low Lopinavir Plasma or Hair Concentrations Explain Second-Line Protease Inhibitor Failures in a Resource-Limited Setting. J Acquir Immune Defic Syndr 56:333–339. doi: 10.1097/QAI.0b013e31820dc0cc
186. Gandhi M, Ameli N, Bacchetti P, Anastos K, Gange SJ, Minkoff H, Young M, Milam J, Cohen MH, Sharp GB, Huang Y, Greenblatt RM (2011) Atazanavir concentration in hair is the strongest predictor of outcomes on antiretroviral therapy. Clin Infect Dis 52:1267–1275. doi: 10.1093/cid/cir131
187. Gandhi M, Ameli N, Bacchetti P, Gange SJ, Anastos K, Levine A, Hyman CL, Cohen M, Young M, Huang Y, Greenblatt RM (2009) Protease inhibitor levels in hair strongly predict virologic response to treatment. AIDS 23:471–478. doi: 10.1097/QAD.0b013e328325a4a9
188. Bernard L, Peytavin G, Vuagnat A, de Truchis P, Perronne C (1998) Indinavir concentrations in hair from patients receiving highly active antiretroviral therapy. Lancet 352:1757–1758. doi: 10.1016/S0140-6736(05)79831-7
189. Bernard L, Vuagnat A, Peytavin G, Hallouin M, Bouhour D, Nguyen TH (2002) Relationship between Levels of Indinavir in Hair and Virologic Response to Highly Active Antiretroviral Therapy. Ann Intern Med 137:656–659. doi: 10.7326/0003-4819-137-8-200210150-00009
190. Gandhi M, Ameli N, Gange S, Anastos K, Cohen M, Young M, Levine A, Minkoff H, Huang Y, Greenblatt R (2010) Concentrations of Efavirenz in Hair Correlate Strongly with 24-Hour Intensive Pharmacokinetic Measurements and with Virologic Outcomes. 17th Conf. Retroviruses Opportunistic Infect. Pap. 604
191. Koss CA, Natureeba P, Mwesigwa J, Cohan D, Nzarubara B, Bacchetti P, Horng H, Clark TD, Plenty A, Ruel TD, Achan J, Charlebois ED, Kamya MR, Havlir D V, Gandhi M (2015) Hair concentrations of antiretrovirals predict viral suppression in HIV-infected pregnant and breastfeeding Ugandan women. AIDS 29:825–830. doi: 10.1097/qad.0000000000000619
192. Baxi SM, Greenblatt RM, Bacchetti P, Jin C, French AL, Keller MJ, Augenbraun MH, Gange SJ, Liu C, Mack WJ, Gandhi M (2015) Nevirapine Concentration in Hair Samples Is a Strong Predictor of Virologic Suppression in a Prospective Cohort of HIV-Infected Patients. PLoS One 10:e0129100. doi: 10.1371/journal.pone.0129100
193. Yan J, Liu J, Su B, Pan X, Wang Z, Wu J, Zhang J, Ruan Y, Hsi J, Liao L, Shao Y, Xing H (2016) Lamivudine Concentration in Hair and Prediction of Virologic Failure and Drug Resistance among HIV Patients Receiving Free ART in China. PLoS One 11:e0154421. doi: 10.1371/journal.pone.0154421
194. Baxi SM, Liu A, Bacchetti P, Mutua G, Sanders EJ, Kibengo FM, Haberer JE, Rooney J, Hendrix CW, Anderson PL, Huang Y, Priddy F, Gandhi M (2015) Comparing the Novel Method of Assessing PrEP Adherence/Exposure Using Hair Samples to Other Pharmacologic and Traditional Measures. J Acquir Immune Defic Syndr 68:13–20. doi:

10.1097/QAI.0000000000000386

195. Gandhi M, Glidden D V, Liu A, Anderson PL, Horng H, Defechereux P, Guanira J V, Grinsztejn B, Chariyalertsak S, Bekker LG, Grant RM (2015) Strong correlation between concentrations of tenofovir (TFV) emtricitabine (FTC) in hair and TFV diphosphate and FTC triphosphate in dried blood spots in the iPrEx open label extension: Implications for pre-exposure prophylaxis adherence monitoring. J Infect Dis 212:1402–1406. doi: 10.1093/infdis/jiv239
196. Liu AY, Yang Q, Huang Y, Bacchetti P, Anderson PL, Jin C, Goggin K, Stojanovski K, Grant R, Buchbinder SP, Greenblatt RM, Gandhi M (2014) Strong Relationship between Oral Dose and Tenofovir Hair Levels in a Randomized Trial: Hair as a Potential Adherence Measure for Pre-Exposure Prophylaxis (PrEP). PLoS One 9:e83736. doi: 10.1371/journal.pone.0083736
197. Huang Y, Gandhi M, Greenblatt RM, Gee W, Lin ET, Messenkoff N (2008) Sensitive analysis of anti-HIV drugs, efavirenz, lopinavir and ritonavir, in human hair by liquid chromatography coupled with tandem mass spectrometry. Rapid Commun Mass Spectrom 22:3401–3409. doi: 10.1002/rcm.3750
198. Huang Y, Yang Q, Yoon K, Lei Y, Shi R, Gee W, Lin ET, Greenblatt RM, Gandhi M (2011) Microanalysis of the antiretroviral nevirapine in human hair from HIV-infected patients by liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 401:1923–1933. doi: 10.1007/s00216-011-5278-7
199. Shah SAB, Mullin R, Jones G, Shah I, Barker J, Petroczi A, Naughton DP (2013) Simultaneous analysis of antiretroviral drugs abacavir and tenofovir in human hair by liquid chromatography-tandem mass spectrometry. J Pharm Biomed Anal 74:308–313. doi: 10.1016/j.jpba.2012.10.023
200. ter Heine R, Beijnen J, Huitema A (2009) Bioanalytical issues in patient-friendly sampling methods for therapeutic drug monitoring: focus on antiretroviral drugs. Bioanalysis 1:1329–1338. doi: 10.4155/bio.09.124
201. Duval X, Peytavin G, Breton G, Ecobichon J-L, Descamps D, Thabut G, Leport C (2007) Hair versus plasma concentrations as indicator of indinavir exposure in HIV-1-infected patients treated with indinavir/ritonavir combination. AIDS 21:106–108. doi: 10.1097/QAD.0b013e3280118486
202. Röhrich CR, Drögemöller BI, Ikediobi O, van der Merwe L, Grobbelaar N, Wright GEB, McGregor N, Warnich L (2016) CYP2B6*6 and CYP2B6*18 Predict Long-Term Efavirenz Exposure Measured in Hair Samples in HIV-Positive South African Women. AIDS Res Hum Retroviruses 32:529–538. doi: 10.1089/aid.2015.0048
203. Pragst F, Suesse S, Salomone A, Vincenti M, Cirimele V, Hazon J, Tsanaclis L, Kingston R, Sporkert F, Baumgartner MR (2017) Commentary on Current Changes of the SoHT 2016 Consensus on Alcohol Markers in Hair and Further Background Information. Forensic Sci Int. doi: 10.1016/j.forsciint.2017.07.023
204. Salomone A, Baumgartner MR, Lombardo T, Alladio E, Di Corcia D, Vincenti M (2016) Effects of various sample pretreatment procedures on ethyl glucuronide quantification in hair samples: Comparison of positivity rates and appraisal of cut-off values. Forensic Sci Int 267:60–65. doi: 10.1016/j.forsciint.2016.08.012
205. Ettlinger J, Kirchen L, Yegles M (2014) Influence of thermal hair straightening on ethyl glucuronide content in hair. Drug Test Anal 6:74–77. doi: 10.1002/dta.1648
206. Tsanaclis L, Kingston R, Wicks J (2009) Testing for alcohol use in hair: is ethyl glucuronide (EtG) stable in hair? Ann Toxicol Anal 21:67–71. doi: 10.1051/ata/2009038
207. Agius R, Ferreira LM, Yegles M (2012) Can ethyl glucuronide in hair be determined only in 3cm hair strands? Forensic Sci Int 218:3–9. doi: 10.1016/j.forsciint.2011.10.001
208. Food and Drug Administration (2001) Guidance for Industry: Bioanalytical Method Validation. Rockville, MD

209. Food and Drug Administration (2013) Draft Guidance: Guidance for Industry: Bioanalytical Method Validation. Rockville, MD
210. European Medicines Agency (2012) Guideline on bioanalytical method validation. London, UK
211. European Medicines Agency (2012) Reflection paper for laboratories that perform the analysis or evaluation of clinical trial samples. London, UK
212. Mueller A, Jungen H, Iwersen-Bergmann S, Raduenz L, Lezius S, Andresen-Streichert H (2017) Determination of ethyl glucuronide in human hair samples: A multivariate analysis of the impact of extraction conditions on quantitative results. Forensic Sci Int 271:43–48. doi: 10.1016/j.forsciint.2016.12.011
213. Bossers LCAM, Paul R, Berry AJ, Kingston R, Middendorp C, Guwy AJ (2014) An evaluation of washing and extraction techniques in the analysis of ethyl glucuronide and fatty acid ethyl esters from hair samples. J Chromatogr B 953–954:115–119. doi: 10.1016/j.jchromb.2014.01.049
214. Cabarcos P, Hassan HM, Tabernero MJ, Scott KS (2013) Analysis of ethyl glucuronide in hair samples by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). J Appl Toxicol 33:638–643. doi: 10.1002/jat.1791
215. Turfus SC, Beyer J, Gerostamoulos D, Drummer OH (2013) A comparison of the performance of quality controls prepared from spiked, fortified and authentic hair for ethyl glucuronide analysis. Forensic Sci Int 232:60–66. doi: 10.1016/j.forsciint.2013.07.003
216. Matuszewski BK, Constanzer ML, Chavez-Eng CM (2003) Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC−MS/MS. Anal Chem 75:3019–3030. doi: 10.1021/ac020361s
217. Matuszewski BK (2006) Standard line slopes as a measure of a relative matrix effect in quantitative HPLC-MS bioanalysis. J Chromatogr B 830:293–300. doi: 10.1016/j.jchromb.2005.11.009
218. Binz TM, Baumgartner MR, Kraemer T (2014) The influence of cleansing shampoos on ethyl glucuronide concentration in hair analyzed with an optimized and validated LC-MS/MS method. Forensic Sci Int 244:20–24. doi: 10.1016/j.forsciint.2014.07.021
219. Sachs H (1997) Quality control by the Society of Hair Testing. Forensic Sci Int 84:145–150. doi: 10.1016/S0379-0738(96)02057-9
220. Kader R, Seedat S, Koch J, Parry C (2012) A preliminary investigation of the AUDIT and DUDIT in comparison to biomarkers for alcohol and drug use among HIV-infected clinic attendees in Cape Town, South Africa. Afr J Psychiatry 15:346–351. doi: 10.4314/ajpsy.v15i5.43
221. Stewart SH, Koch DG, Willner IR, Randall PK, Reuben A (2013) Hair Ethyl Glucuronide is Highly Sensitive and Specific for Detecting Moderate-to-Heavy Drinking in Patients with Liver Disease. Alcohol Alcohol 48:83–87. doi: 10.1093/alcalc/ags109
222. Kharbouche H, Faouzi M, Sanchez N, Daeppen JB, Augsburger M, Mangin P, Staub C, Sporkert F (2012) Diagnostic performance of ethyl glucuronide in hair for the investigation of alcohol drinking behavior: a comparison with traditional biomarkers. Int J Legal Med 126:243–250. doi: 10.1007/s00414-011-0619-9
223. Boscolo-Berto R, Favretto D, Cecchetto G, Vincenti M, Kronstrand R, Ferrara SD, Viel G (2014) Sensitivity and Specificity of EtG in Hair as a Marker of Chronic Excessive Drinking. Ther Drug Monit 36:560–575. doi: 10.1097/FTD.0000000000000063
224. Kintz P (2004) Value of hair analysis in postmortem toxicology. Forensic Sci Int 142:127–134. doi: 10.1016/j.forsciint.2004.02.027
225. Barroso M, Gallardo E (2014) Hair analysis for forensic applications: is the future bright? Bioanalysis 6:1–3. doi: 10.4155/bio.13.291
226. Dada S, Erasmus J, Burnhams N, Parry C, Bhana A, Timol F, Fourie D, Kitshoff D, Nel E,

Weimann R (2016) Monitoring alcohol, tobacco and other drug abuse trends July 1996 - December 2015. South African Community Epidemiology Network on Drug Use (SACENDU) Research Brief

227. Scientific Working Group for Forensic Toxicology (2013) Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology. J Anal Toxicol 37:452–474. doi: 10.1093/jat/bkt054
228. Trullols E, Ruisánchez I, Rius FX (2004) Validation of qualitative analytical methods. Trends Anal Chem 23:137–145. doi: 10.1016/S0165-9936(04)00201-8
229. Jiménez C, Ventura R, Segura J (2002) Validation of qualitative chromatographic methods: strategy in antidoping control laboratories. J Chromatogr B 767:341–351. doi: 10.1016/S1570-0232(01)00593-1
230. Peters FT, Drummer OH, Musshoff F (2007) Validation of new methods. Forensic Sci Int 165:216–224. doi: 10.1016/j.forsciint.2006.05.021
231. Wainberg MA, Friedland G (1998) Public Health Implications of Antiretroviral Therapy and HIV Drug Resistance. JAMA 279:1977–1983. doi: 10.1001/jama.279.24.1977
232. Sinxadi PZ, Leger PD, McIlleron HM, Smith PJ, Dave JA, Levitt NS, Maartens G, Haas DW (2015) Pharmacogenetics of plasma efavirenz exposure in HIV-infected adults and children in South Africa. Br J Clin Pharmacol 80:146–156. doi: 10.1111/bcp.12590
233. Gandhi M, Greenblatt RM, Bacchetti P, Jin C, Huang Y, Anastos K, Cohen M, DeHovitz JA, Sharp GB, Gange SJ, Liu C, Hanson SC, Aouizerat B (2012) A Single-Nucleotide Polymorphism in CYP2B6 Leads to >3-Fold Increases in Efavirenz Concentrations in Plasma and Hair Among HIV-Infected Women. J Infect Dis 206:1453–1461. doi: 10.1093/infdis/jis508
234. Gandhi M, Mwesigwa J, Aweeka F, Plenty A, Charlebois E, Ruel TD, Huang Y, Clark T, Ades V, Natureeba P, Luwedde FA, Achan J, Kamya MR, Havlir D V, Cohan D (2013) Hair and Plasma Data Show That Lopinavir, Ritonavir, and Efavirenz All Transfer From Mother to Infant In Utero, But Only Efavirenz Transfers via Breastfeeding. J Acquir Immune Defic Syndr 63:578–584. doi: 10.1097/QAI.0b013e31829c48ad
235. Orrell C, Cohen K, Leisegang R, Bangsberg DR, Wood R, Maartens G (2017) Comparison of six methods to estimate adherence in an ART-naïve cohort in a resource-poor setting: which best predicts virological and resistance outcomes? AIDS Res Ther 14:1–20. doi: 10.1186/s12981-017-0138-y
236. Gross R, Bellamy SL, Ratshaa B, Han X, Steenhoff AP, Mosepele M, Bisson GP (2017) Effects of sex and alcohol use on antiretroviral therapy outcomes in Botswana: a cohort study. Addiction 112:73–81. doi: 10.1111/add.13538
237. Jaquet A, Ekouevi DK, Bashi J, Aboubakrine M, Messou E, Maiga M, Traore HA, Zannou MD, Guehi C, Ba-Gomis FO, Minga A, Allou G, Eholie SP, Bissagnene E, Sasco AJ, Dabis F (2010) Alcohol use and non-adherence to antiretroviral therapy in HIV-infected patients in West Africa. Addiction 105:1416–1421. doi: 10.1111/j.1360-0443.2010.02978.x
238. Ewing JA (1984) Detecting Alcoholism: The CAGE Questionnaire. JAMA 252:1905–1907. doi: 10.1001/jama.1984.03350140051025
239. Mayfield D, Mcleod G, Hall P (1974) The CAGE Questionnaire: Validation of a New Alcoholism Screening Instrument. Am J Psychiatry 131:1121–1123.
240. Orrell C, Cohen K, Mauff K, Bangsberg DR, Maartens G, Wood R (2015) A Randomized Controlled Trial of Real-Time Electronic Adherence Monitoring With Text Message Dosing Reminders in People Starting First-Line Antiretroviral Therapy. J Acquir Immune Defic Syndr 70:495–502. doi: 10.1097/QAI.0000000000000770
241. Orrell C, Bienczak A, Cohen K, Bangsberg D, Wood R, Maartens G, Denti P (2016) Effect of mid-dose efavirenz concentrations and CYP2B6 genotype on viral suppression in patients on first-line antiretroviral therapy. Int J Antimicrob Agents 47:466–472. doi: 10.1016/j.ijantimicag.2016.03.017

242. Wisepill Technologies. https://www.wisepill.com. Accessed 6 Jun 2017
243. Samet JH, Phillips SJ, Horton NJ, Traphagen ET, Freedberg KA (2004) Detecting Alcohol Problems in HIV-Infected Patients: Use of the CAGE Questionnaire. AIDS Res Hum Retroviruses 20:151–155. doi: 10.1089/088922204773004860
244. Buchsbaum DG, Buchanan RG, Centor RM, Schnoll SH, Lawton MJ (1991) Screening for Alcohol Abuse Using CAGE Scores and Likelihood Ratios. Ann Intern Med 115:774–777. doi: 10.7326/0003-4819-115-10-774
245. Holzinger ER, Grady B, Ritchie MD, Ribaudo HJ, Acosta EP, Morse GD, Gulick RM, Robbins GK, Clifford DB, Daar ES, McLaren P, Haas DW (2012) Genome-wide association study of plasma efavirenz pharmacokinetics in AIDS Clinical Trials Group protocols implicates several CYP2B6 variants. Pharmacogenet Genomics 22:858–867. doi: 10.1097/FPC.0b013e32835a450b
246. Mbulaiteye S, Ruberantwari A, Nakiyingi J, Carpenter L, Kamali A, Whitworth J (2000) Alcohol and HIV: a study among sexually active adults in rural southwest Uganda. Int J Epidemiol 29:911–915. doi: 10.1093/ije/29.5.911
247. Farley J, Miller E, Zamani A, Tepper V, Morris C, Oyegunle M, Lin M, Charurat M, Blattner W (2010) Screening for Hazardous Alcohol Use and Depressive Symptomatology Among HIV-Infected Patients in Nigeria: Prevalence, Predictors, and Association With Adherence. J Int Assoc Physicians AIDS Care 9:218–226. doi: 10.1177/1545109710371133
248. Ortego C, Huedo-Medina TB, Santos P, Rodríguez E, Sevilla L, Warren M, Llorca J (2012) Sex differences in adherence to highly active antiretroviral therapy: A meta-analysis. AIDS Care 24:1519–1534. doi: 10.1080/09540121.2012.672722
249. Beumer JH, Bosman IJ, Maes RA (2001) Hair as a biological specimen for therapeutic drug monitoring. Int J Clin Pract 55:353–357.
250. Hickey MD, Salmen CR, Tessler RA, Omollo D, Bacchetti P, Magerenge R, Mattah B, Salmen MR, Zoughbie D, Fiorella KJ, Geng E, Njoroge B, Jin C, Huang Y, Bukusi EA, Cohen CR, Gandhi M (2014) Antiretroviral Concentrations in Small Hair Samples as a Feasible Marker of Adherence in Rural Kenya. J Acquir Immune Defic Syndr 66:311–315. doi: 10.1097/QAI.0000000000000154
251. Olds P, Kiwanuka J, Nansera D, Huang Y, Bacchetti P, Jin C, Gandhi M, Haberer JE (2015) Assessment of HIV antiretroviral therapy adherence by measuring drug concentrations in hair among children in rural Uganda. AIDS Care 27:327–332. doi: 10.1080/09540121.2014.983452
252. Loussouarn G, El Rawadi C, Genain G (2005) Diversity of hair growth profiles. Int J Dermatol 44:6–9. doi: 10.1111/j.1365-4632.2005.02800.x
253. Loussouarn G (2001) African hair growth parameters. Br J Dermatol 145:294–297. doi: 10.1046/j.1365-2133.2001.04350.x
254. Khumalo NP, Stone J, Gumedze F, McGrath E, Ngwanya MR, de Berker D (2010) “Relaxers” damage hair: Evidence from amino acid analysis. J Am Acad Dermatol 62:402–408. doi: 10.1016/j.jaad.2009.04.061
255. Coetzee B, Kagee A, Tomlinson M, Warnich L, Ikediobi O (2012) Reactions, beliefs and concerns associated with providing hair specimens for medical research among a South African sample: a qualitative approach. Future Virol 7:1135–1142. doi: 10.2217/fvl.12.100