Development and Partial Validation of a Method for the Quantification of Benzodiazepines and Antidepressants in Whole Blood, Serum and Urine by Liquid Chromatography - Tandem Mass Spectrometry

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JANKE P E E S

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Declaration

I, Janke Pieters, hereby declare that the work on which this minor dissertation is based on is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Date: 01 May 2015

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Abbreviations

%Accuracy	The concentration of the analyte found against the nominal concentration expressed as a percentage.
%CV	Percentage Coefficient of Variation. Used as expression of 'Precision' of an analytical procedure.
AA	ammonium acetate
ACN	acetonitrile
APCI	atmospheric pressure chemical ionization
DAD	diode array detector
DUID	driving under the influence of drugs
EMEA	European Medicines Agency
ESI	electrospray ionization
FA	formic acid
FDA	Food and Drug Administration
FIA	flow injection analysis
GABA	gamma-aminobutyric acid
GC-MS	gas chromatography – mass spectrometry
GUS	general unknown screening
H QC	high quality control (1600 ng/mL)
HCl	hydrogen chloride
HCX	mixed-mode sorbent with hydrophobic and cation exchange

properties

- HLB — polymer sorbent with hydrophilic and lipophilic properties
- HPLC — high performance liquid chromatography
- IS — internal standard
- L QC — low quality control (45 ng/mL)
- LC-MS — liquid chromatography – mass spectrometry
- LC-MS/MS — liquid chromatography – tandem mass spectrometry
- LLE — liquid liquid extraction
- LLOQ — lower limit of quantification
- LOD — limit of detection
- LOQ — limit of quantification
- M QC — medium quality control (800 ng/mL)
- MeOH — methanol
- MEs — matrix effects
- MRM — multiple reaction monitoring
- n — number of determinations
- PP — protein precipitation
- Rt — retention time
- SANAS — South African National Accreditation System
- SIM — selected-ion monitoring
- S/N — signal-to-noise ratio
- SOP — standard operating procedure
- SPE — solid phase extraction
- SRM — selected reaction monitoring
- SSRI — selective serotonin re-uptake inhibitors
- STA — systematic toxicological analysis
- STD — calibration standard
- SWGTOX — Scientific Working Group for Forensic Toxicology
- TCAs — tricyclic antidepressants
- TOF — time-of-flight
- UPLC — ultra-performance liquid chromatography
- Q — single quadrupole
- QC — quality control standard
- QQQ/LIT — hybrid triple quadrupole/linear ion trap
- QQQ — triple quadrupole
- WPBTS — Western Province Blood Transfusion Services

Units of Measurement

- cps counts per second
- Da Daltons
- °C degrees Celsius
- m/z mass to charge ratio
- µg/mL microgram per millilitre
- µm micrometer
- µL microliter
- mg/mL milligram per millilitre
- mm milimeter
- mM milimolar
- min minutes
- ng/mL nanogram per millilitre
- % percentage
- rmp revolutions per minute
- s seconds
- V voltage

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1. Introduction

Immunoassay techniques are frequently used to determine the presence of drugs of abuse in bodily fluids. Immunoassay systems are advantageous due to its high-throughput capability with minimal sample preparation (Eichhorst*et al*., 2012). A shortcoming of immunoassay-based drug testing is that it is subject to false-positives. The technique has a reduced sensitivity due to a high degree of cross-reactivity that can exist for each species within a class of drugs (Eichhorst*et al.*, 2012). Results could thus show the presence of metabolites of a drug but the detection of exactly which form of that drug is present may be inadequate. Confirmatory testing using another analytical tool is thus required to determine the actual analyte and its concentration in the body fluid that is analysed.

Numerous analytical methods have been published for the screening and quantification of drugs of abuse in the toxicology field. Quantification of drugs in bio-samples is a highly important criterion for proficient toxicological assessment and consultation in clinical and forensic toxicology. Due to the serious consequences linked to forensic and clinical toxicological results, a selective and reliable method of quantification is sought-after (Maurer, 2005). Liquid chromatography coupled with mass spectrometry (LC-MS) has become the most robust analytical tool for identification and quantification of drugs and their metabolites in various biological samples (Roškar&Lušin, 2012). This project aims to develop and validate quantitative LC-MS based assays for a number of drugs of abuse.

Method validation can be described as the process whereby the performance characteristics of an analytical method are ascertained via laboratory studies to meet the requirements for its intended analytical applications (SANAS TG 41-01:2008). ISO/IEC 17025:2005 documents the general requirements for the competence of any laboratory to carry out tests and it necessitates that “all technical procedures used by a forensic science laboratory must be fully validated before being used on casework” (SANAS TG 41-01:2008). Validation of an analytical method is thus of the upmost importance for a laboratory to be able to confidently employ the method for regular testing.

Benzodiazepines, opiates and some antidepressant drugs are among some of the most frequently encountered analytes identified in biological matrices during routine toxicological analysis. These prescription drug classes have been found to be in close association with

drug dependence amongst patients owing to some of the satisfying effects they induce, consequently making them popular amongst abusers (White &Taverner, 1997).

Benzodiazepines are frequently misused since it heightens the affinity of $GABA_A$ receptors in the nervous systems for its endogenous ligand, the neurotransmitter gamma-aminobutyric acid (GABA) leading to sedative and anti-anxiety effects (Page *et al.*, 2002). Additionally, the amnestic properties of benzodiazepines make them desirable agents for use in a drug-facilitated sexual assault, therefore making this drug class an important forensic toxicological focus (Morris-Kukoski, Schaff, &Reda, 2012).

Opiates such as codeine and morphine which can be found in nature as well as the semi-synthetic hydrocodone or hydromorphone have long been used as indispensable pain controlling agents (Jickells&Negrusz, 2008). Opiates are thus used chronically to treat pain and it furthermore possesses euphoric and addictive qualities – making them a common drug class that is illicitly abused (Eichhorst*et al.*, 2012).

Antidepressants comprise a diverse group of compounds including the traditional tricyclic antidepressants and the newer generation of antidepressants, the selective serotonin re-uptake inhibitors (SSRIs). These antidepressants are also abused. Tricyclic antidepressants have adverse side-effects such as an increase in suicidal thoughts and too high doses can cause “cardiac disturbances, respiratory depression, metabolic acidosis, convulsions and coma” (Jickells&Negrusz, 2008). SSRIs, including citalopram, are preferred prescription antidepressants since they are less toxic. Serotonergic syndrome and other risks such as cardiac conductive failures and rhabdomyolysis that are associated with the use of SSRIs are however taken too lightly (Jickells&Negrusz, 2008).

Certain sedatives, analgesics and antidepressants as described above have been implicated in impaired driving cases (Jickells&Negrusz, 2008). “Driving has been shown to be affected in driving simulators, in on-road driving studies and from epidemiological and anecdotal reports for many drugs, including opioids and benzodiazepines as reviewed in two special issues of Forensic Science Reviews (vol. 14, 2002, and vol. 15, 2003)” (Jickells&Negrusz, 2008). It is clear that driving under the influence of drugs (DUID) cases are not solely associated with only illegal drugs. Moreover, an increased intensity of driving impairment is

caused by the use of alcohol in combination with recreational or prescription drugs (Jickells&Negrusz, 2008).

It is clear that abuse of these types of drugs is not without risk: other than the causation of driving impairment which can have its own severe consequences, abuse can lead to irreversible damage to a person's system and moreover fatal intoxications have been reported. For these reasons screening procedures are needed in both forensic and clinical toxicology to allow for the reliable determination of substances in biological matrices.

2. Literature Review

Systematic Toxicological Screening

General unknown screening (GUS) or systematic toxicological analysis (STA) is a procedure to screen for a wide range of possible xenobiotics present in biological fluids in cases of unknown poisoning or intoxication. GUS usually involves the initial evaluation of samples via immunochemical techniques to provide a prompt binary yes/no result which indicates the presence or absence of a target analyte above a specified threshold (Sturm, 2005). Should a positive result for one or more compound classes or target substances arise then the sample is to be analysed by a confirmatory analytical method.

Aside from the initial immunoassays for drugs of abuse, the general unknown screening procedure in most clinical and forensic toxicology laboratories also involves chromatographic procedures which are coupled to specific detectors. It is desirable for the GUS analytical method to identify a large number of relevant compounds in an unambiguous manner. For this purpose gas chromatography coupled to mass spectrometry (GC-MS) or high performance liquid chromatography (HPLC) with diode array detection (DAD) are primary employed by most laboratories (Sturm, 2005). These methods can however not adequately recognise all possible toxic analytes. Gas chromatography can strictly be used for the analysis of volatile, nonpolar and thermally stable compounds as it requires compound derivatization. In cases where certain polar compounds have limited UV absorbance for instance, GC-MS nor HPLC-DAD would be sufficient for detection (Sturm, 2005).

LC-MS and LC-MS/MS have in recent years become an imperative technique as it allows for a larger range of compounds to be compliant with mass spectrometric detection. Aqueous samples and hydrophilic, thermolabile and non-volatile analytes can be analysed with this technique (Peters, 2011). LC-MS-based screening procedures can involve triple quadrupole, ion trap, or hybrid mass spectrometers to yield ion spectra that are searched against libraries of reference mass spectra which have been recorded on the same apparatus at a prior stage (Peters, 2011). Another approach is the use of high resolution mass-spectrometry with benchtop time-of-flight (TOF) detectors. In this instance the identification of compounds involves the comparison of measured accurate masses in the specimen to accurate mass databases of toxicologically relevant compounds (Peters, 2011).

Once it is known what analytes are present in the biosamples that were screened, a LC-MS/MS quantitation method that have been developed specifically for these known analytes can be utilised to determine the exact concentrations which allows for interpretation for forensic toxicology cases.

Target Analytes

It is sought-after in forensic toxicology to develop procedures that allow for the concurrent analysis of multiple relevant drugs within a certain drug class or of numerous drug classes that are closely related and commonly abused (Peters, 2011). This is due to the conservation of both time and resources that such multi-analyte procedures can sanction during method development and validation (Peters, 2011). Furthermore it results in a smaller number of methods to be created by the laboratory to cover a broad spectrum of analytes (Peters, 2011).

There have been a number of recent publications that report the successful simultaneous screening of a large number of analytes in biological matrices by the use of LC-MS/MS. Lee (2013) published a robust method for the concurrent screening of over 170 drugs of abuse commonly found in emergency cases in urine samples via LC-MS/MS. Di Rago*et al.* presented a LC-MS/MS method that has proven to be applicable for the quantification of 132 acidic and neutral analytes in blood in March 2014.

Biosamples

Blood, serum/plasma and urine are the typical specimens for toxicological analysis and the choice of specimen is regularly influenced by the forensic or clinical situation. Whole blood, often interchangeable with plasma or serum in most methods, is the most analysed type of specimen in forensic toxicology due to its ease of collection from deceased or unconscious individuals (Sturm, 2005). Blood matrices are important for quantitative measurements as the drug concentration in blood, plasma or serum tends to show the best correlation with pharmacologic effects (Flanagan *et al.*, 2007).

Urine is the most frequent specimen used in clinical situations. It is easier and less invasive to obtain a large volume of urine compared to a blood sample from a patient that has damaged veins and additionally human urine imposes less of a health risk to laboratory staff (Flanagan *et al.*, 2007). The value of urine samples is that drug and drug metabolite concentrations tend to be much higher than in blood. It must however be remarked that certain drug classes, for example benzodiazepines, are considerably metabolized before it is excreted. In such cases blood plasma should be the specimen of choice for the detection of the parent compound (Flanagan *et al.*, 2007). It is more difficult to link urine concentrations to pharmacologic effects.

There are a number of other less conventional biological specimens that have also been collected and analysed for toxicological purposes which include: hair, oral fluid (saliva), umbilical cord, placenta and meconium, tissue (brain and adipose), sweat, breath and nail clippings (Saito *et al.*, 2011).

Sample Preparation

Pre-treatment of samples to remove interfering compounds, such as lipids, proteins, salts, and the like that are present within the biological matrices is needed for instrument soiling and to prevent clogging of the analytical column (Roškar&Lušin, 2012). Problems such as interfering peaks might arise with the use of spectroscopic detection (fluorescence/UV-absorbance) if samples are insufficiently treated. This phenomenon is less likely to be an issue when using LC-MS/MS procedures and sample preparation can be relatively straightforward (Roškar&Lušin, 2012).

There are various types of sample workup procedures. Protein precipitation (PP), liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are some of the most broadly used sample preparation techniques (Roškar&Lušin, 2012).Selection of the most appropriate sample preparation technique is highly dependent on the sample size and matrix as well as analyte characteristics and their expected concentrations (Roškar&Lušin, 2012). Protein precipitation has found to be a simple work-up for matrices like blood, serum or plasma (Peters, 2011).

Urine contains less interfering matrix compounds. Simple dilution for preparation of urine samples have been published before with a dilution factor ranging from five-fold up to 50-fold (Anderssonetal., 2008; Svenssonetal., 2007; Gustavssonetal., 2007). Eichhorstet al. (2009) did a ten-fold dilution of urine after a hydrolysis step was performed.

An enzymatic hydrolysis step may be needed during urine sample preparation for the cleavage of glucuronic or sulphuric acid conjugates to compounds that are more easily measurable by sensitive GC-MS (Peters, 2011; Sturm, 2005). This kind of treatment is not necessarily needed for analysis by LC-MS/MS since it can directly examine conjugated compounds (Peters, 2011).

Liquid Chromatography Separation

Separation of compounds by the use of gas chromatography has long been a method of choice by most analytical laboratories but LC-MS have a primary advantage over GC-MS in that the technique has the capacity to analyse a much wider range of compounds as no derivatization step is needed. Another advantage of LC-MS is the use of short columns and gradient elution to give short analysis times and a reduced eluent consumption (Flanagan *et al.*, 2007).

There exist a wide range of possible mobile-phase properties together with the choice of numerous, significantly different kinds of stationary phases when it comes to the separation of analytes by liquid chromatography. During method development these are some variables to consider experimenting with to determine what combination of phases yields the best chromatography.

The table below contains some published examples of procedures for the determination of multiple analytes in the benzodiazepine, opiate or antidepressant drug classes from blood or urine samples by liquid chromatography-tandem mass spectrometry (Peters, 2011).

Table 1:Procedures for the determination of multiple analytes in biofluids by LC-MS/MS

Analytes	Sample	Work-Up	Stationary Phase	Mobile Phase	Detection Mode	Apparatus Type	Reference
25 Opioid drugs	Urine, Blood	Enzymatic hydrolysis, LLE	Gemini C18 (100×2mm, 3 µm)	Gradient, aq. AA and ACN with 0.1% FA	ESI+, MRM (2)	QQQ/LIT	Gergov*et al.* (2009)
Morphine, codeine, ethylmorphine and glucuronides, 6-acetylmorphine	Urine	Dilution	Luna C18 (100×2mm, 3 µm)	Gradient, water and ACN with 25mM FA	ESI+,SRM (2)	QQQ	Gustavsson*et al.* (2007)
Opiates and cocaine	Urine	SPE (HCX)	UPLC BEH C18 (50×2.1mm, 1.7 µm)	Gradient, 2 mM ammonium bicarbonate and MeOH	ESI+,MRM (1)	QQQ	Berg *et al.* (2009)
6-acetylmorphine glucuronides of morphine, codeine, ethylmorphine	Urine	SPE (HLB)	Luna C18 (100×2.0mm, 3 µm)	Gradient, 25 mM FA in water and ACN	ESI+,SIM (2)	Q	Svensson*et al.* (2007)
Some Opiates	Blood	SPE	Synergi Polar-RP (150×2 mm, 4 µm)	Gradient, 1 mM AF with 0.1% FA and ACN	ESI+,MRM (2–3)	QQQ	Taylor & Elliott (2009)
Midazolam, morphine, and metabolites	Plasma	SPE (HLB)	Aquity UPLC BEH C18 (2.1×100mm, 1.7 µm)	Gradient, water and MeOH with 0.1% FA	ESI+, MRM, 1 transition per analyte	QQQ	Ahsman*et al.* (2010)
Alprazolam, flunitrazepam, metabolites	Blood	96 well SPE (HLB)	XBridge Shield (100×2.1 mm, 3.5 µm)	Gradient, 20 mM acetate buffer and ACN	APPI, SIM (1)	Q	Marchi*et al.* (2009)
14 antidepressants and their metabolites	Plasma	On-line SPE	Gemini C18 guard column (4mm×2.0 mm, 5um)	Gradient, 10mM ammonium hydrogencarbonate (pH 10)	ESI+, MRM	Q	De Castro *et al.* (2007)

HCX, mixed-mode sorbent with hydrophobic and cation exchange properties; HLB, polymer sorbent with hydrophilic and lipophilic properties; ACN, acetonitrile; FA, formic acid; AA, ammonium acetate; MeOH, methanol; ESI, electrospray ionization; MRM, multiple reaction monitoring; QQQ/LIT, hybrid triple quadrupole/linear ion trap; QQQ, triple quadrupole

3. Justification

Liquid chromatography- tandem mass spectrometry (LC-MS/MS) has shown to be an extremely useful and increasingly important technique in forensic and clinical toxicology (Pitt, 2013). It is however still paramount to validate individual LC-MS/MS procedures to guarantee their usefulness and reliability for their envisioned purposes (Polettini, 2006). Once this is fully established, the benefit of incorporating the validated techniques into routine analysis can successfully be achieved (EMEA/CHMP/EWP/192217/2009).

The validation of LC-MS/MS procedures furthermore requires the consideration of certain features apart from the typical recommendations suggested for bio-analytical method validation. Possible matrix effects (MEs) which include ion suppression or enhancement for example is particularly unique to the LC-MS/MS technique in the electrospray ionization(ESI) mode and should be investigated during validation. Stability is another important validation parameter for an LC-MS/MS technique that is to be used in forensic and/or clinical toxicology. (Polettini, 2006)

Validation data for an LC-MS/MS quantification procedure is essential to establish whether the procedure can determine the analytes of interest in biological matrices in a fixed and reliable manner. In order for the application of the bio-analytical method, it should be well characterised, fully validated and documented to a satisfactory standard (EMEA/CHMP/EWP/192217/2009). This will aid in warranting the generation of reliable results. Steadfast analytical data is crucial for the proper interpretation of toxicological findings. Erroneous results can be contested in court and furthermore it could result in unfair legal penalties for the defendant or even incorrect treatment of patients which in itself can have severe consequences (Polettini, 2006). It is thus clear that validation of routine analytical methods is extremely vital. As stated by Polettini (2006), method validation is fundamental to important matters such as accreditation and quality management in the field of analytical toxicology.

A quantitative method testing multiple analytes which this project aims to develop is desirable compared to single-analyte approaches as it is practically and economically more feasible. Combining a number of analytes in a single method allows for streamlining of routine practices within the laboratory and a decreased specimen volume can be used for

such analysis. The quantitative nature of the findings that will be produced will allow for interpretation of any suspected drug-related deaths as well as drug overdose cases.

4. Objectives

The aim of this project is to develop a single quantification method for certain benzodiazepines, opiates and antidepressants in whole blood, serum and urine by LC-MS/MS and to consequently validate the analytical method for official use in the Division of Pharmacology at the University of Cape Town.

5. Research plan

Validation Parameters

Validation guidelines for bio-analytical methods provided by the European Medicines Agency (EMEA) in combination with those provided by the U.S. Food and Drug Administration (FDA) are used as international recommendations for the validation of the quantification method to be characterised by this project. Based on these guidelines, a Validation log document has been derived for the use in the Division of Pharmacology at the University of Cape Town that states possible validation parameters that need to be achieved. The procedures to be followed to achieve each parameter will be stipulated as well as the acceptance criteria for each parameter.

Table 2:Descriptions of the some validation parameters likely to be achieved

Validation Parameters	Definition/Description	Validation Parameters	Definition/Description
Accuracy	The accuracy of an analytical procedure expresses the closeness of the determined value to the value which is accepted either as a conventional true value or an accepted reference value. Accuracy is defined as (determined value/true value) x100%. (EMEA/CHMP/EWP/192217/2009)	**Benchtop Stability**	The stability of analyte under the conditions of sample preparation (e.g. ambient temperature over time needed for sample preparation). (Polettini, 2006)
Precision	The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained under the prescribed conditions. Precision is defined as the ratio of standard deviation/mean (%). (EMEA/CHMP/EWP/192217/2009)	**Autosampler Stability**	Instability can occur not only in the sample matrix, but also in processed samples. It is therefore important also to test the stability of an analyte in the prepared samples under conditions of analysis (e.g. auto-sampler conditions for the expected maximum time of an analytical run). (Polettini, 2006)
Matrix Effects	The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample. (EMEA/CHMP/EWP/192217/2009)	**Freeze Thaw Stability**	As samples are often frozen and thawed, e.g. for re-analysis, the stability of analyte during several freeze/thaw cycles should be evaluated. (Polettini, 2006)
Dilutions	Dilutions of samples should not affect the accuracy and precision. Dilution integrity should be demonstrated by spiking the matrix with an analyte concentration above the ULOQ and diluting this sample with blank matrix. (EMEA/CHMP/EWP/192217/2009)	**Cross Talk**	Interference between SIM channels when either analogues or stable isotope-labelled internal standards are used. (Flanagan *et al*., 2007)
Recovery	Recovery can usually be calculated as the percentage of the analyte response after sample work-up compared with that of a solution containing the analyte at the theoretical maximum concentrations. (Polettini, 2006)	**ISR (Incurred Sample Repeatability)**	The analysis of a portion of the incurred samples to determine whether the original analytical results are reproducible. (EMEA/CHMP/EWP/192217/2009)

Materials

***Reference Standard Stock Solutions*:**

(a) Benzodiazepine Multi-Component Mixture-8 Stock Standard at a concentration of 250 µg/mL, prepared in acetonitrile: alprazolam, clonazepam, diazepam,

flunitrazepam, lorazepam, nitrazepam, oxazepam, temazepam (Cerilliant Corp, Round Rock, TX).

(b) Pain Management Multi-Component Opiate Mixture-13 Stock Standard at a concentration of 10 µg/mL of fentanyl plus 100 µg/mL of buprenorphine, codeine, hydrocodone, hydromorphone, meperidine, (±)-methadone, morphine, oxycodone, oxymorphone, cis-tramadol HCl (as free base), naloxone, naltrexone prepared in methanol (Cerilliant Corp, Round Rock, TX).

(c) Morphine-3-β-D-glucoronide Stock Standard at a concentration of 100 µg/mL prepared in methanol:water (1:1) (Cerilliant Corp, Round Rock, TX).

(d) 6-Acetylmorphine Stock Standard at a concentration of 1.0 mg/ml prepared in acetonitrile (Cerilliant Corp, Round Rock, TX).

(e) Amitriptyline Hydrochloride Stock Standard at a concentration of 1.000 mg/mL (as free base) prepared in methanol (Cerilliant Corp, Round Rock, TX).

(f) Nortriptyline Hydrochloride Stock Standard at a concentration of 1.000 mg/mL (as free base) prepared in methanol (Cerilliant Corp, Round Rock, TX).

(g) Citalopram, Primary Standard at a concentration of 100 µg/mL prepared in methanol (Cerilliant Corp, Round Rock, TX).

Table 3:Summary of the 15 analytes to be included in the quantitation method

Drug Class	Analytes	Drug Class		Analytes
Benzodiazepines	Alprazolam	Opiates		Morphine
	Clonazepam			Codeine
	Diazepam			Morphine-3-β-D-glucoronide
	Flunitrazepam			6-Acetylmorphine
	Lorazepam	Antidepressants	Tricyclic	Amitriptyline
	Nitrazepam			Nortriptyline
	Oxazepam		SSRI	Citalopram
	Temazepam			

Internal standards:

Doxepin-D3 and Diazepam-D5

Authentic samples:

Authentic blood, urine and serum forensic samples which tested positive for the drug classes which tested positive for one or more of the drugs by qualitative mass spectrometry will be used as part of the validation process for the quantitative assay as will patient samples submitted to the Division of Pharmacology for toxicological analysis by immunoassay. The latter samples will be anonymized for purposes of the project. An application to the UCT Research Ethics Committee for use of the samples is attached under the appendices. Samples will be stored at -20°Cfor the purpose of validating the quantitation method. Once validated, the method will be used for quantitative analysis of both forensic samples and for patient samples submitted routinely to the Division of Clinical Pharmacology.

Equipment:

A Shimadzu Prominence High Performance Liquid Chromatography System coupled to an Applied Biosystems/MDS Analytical Technologies API 3200^{TM} Triple Quad MS/MS System will be used for the screening and quantification of biosamples.

Methods

Sample Preparation for Blood and Serum Specimens:

Protein precipitation of low sample volumes (100µl) with acetonitrile.

Sample Preparation for Urine Specimens:

Ten-fold dilution or protein precipitation with acetonitrile.

Liquid Chromatography Conditions:

Gradient elution will be tested with different aqueous mobile phases (e.g. 10mM Ammonium Acetate in water) and organic phases (e.g. 50% methanol:acetonitrile).

Different analytical columns (e.g. C18 columns and PFP columns) will also be experimented with in conjunction with different combinations of mobile phases.

Oven temperatures of about 40°C to 60°C will be considered.

Considering the amount of analytes included in the quantitation method, a longer run time of about 10 minutes will be attempted.

Injection volumes and flow rates will also be tested.

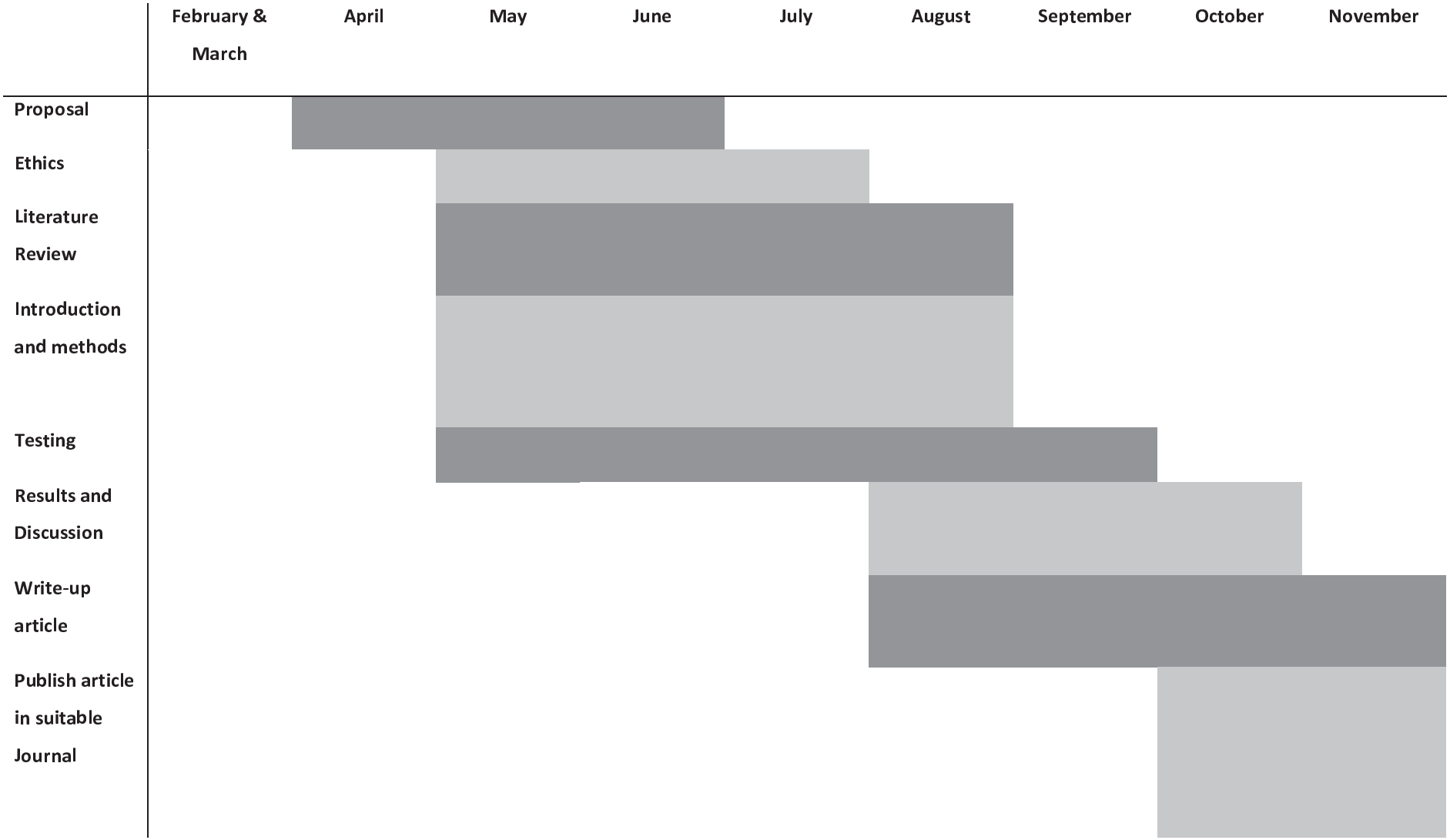
Mass Spectral Conditions:

Positive electrospray ionization (ESI) in MRM mode will be employed. Other mass spectral parameters will also be optimized (e.g. source temperature).

Data Analysis:

Chromatograms will be automatically analysed using Analyst 1.6.1 software.

Timetable


February & March
April
May
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July
August
September
October
November
Proposal
Ethics
Literature Review
Introduction and methods
Testing
Results and Discussion
Write-up article
Publish article in suitable Journal

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Addendum I: Adjustments to project proposal

Some changes were made to the study design described in the research proposalduring the course of the study and are described below.

Validation parameters

Due to time constraints, it was not possible to perform a full validation of the developed LC-MS/MS method in each of the three matrices. For all three matrices, experiments were performed to evaluate the linearity, accuracy and precision of the quantification method. Some extra validation parameters were tested for the method in whole blood which included certain stability experiments as well as testing recovery, specificity and sensitivity.

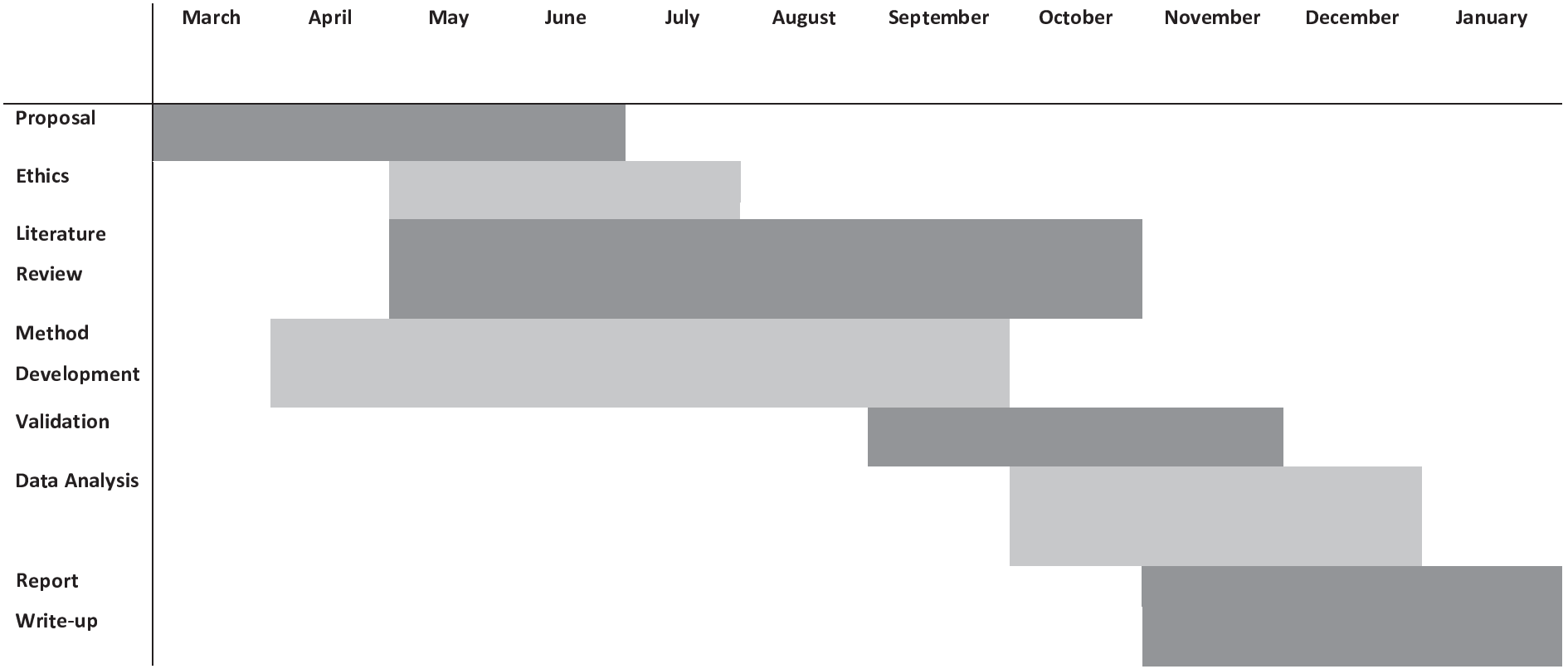
Analytes of interest

During liquid chromatography optimization, it was decided to exclude the four opiate compounds from the method as resulting chromatograms only showed acceptable peak morphology for the respective benzodiazepines and antidepressant analytes. In the very same chromatograms, the peaks observed forthe four opiate analytes did not show desirable results. It was debated that opiates as a drug class might run in a more distinctive manner through the LC-MS/MS system compared to the benzodiazepine and antidepressant drug classes. Spending more time onliquid chromatography development in conjunction with trying different sample preparations might have corrected this but due to limited time this could not be tested.This was an indication of some of the challenges that can arise when aiming to include multiple analytes in a LC-MS/MS method.Since acceptable chromatography was indeed achieved for the other 11 compounds, the focus of the project was to then test validation parameters.

Revised Timeline

Due to divided opportunities to utilize the available LC-MS/MS instrument along with certain unforeseen instrumental lapses that arose, the progress of the project shifted from what was initially planned in the project protocol. An amended timeline of the progress of the research project follows below.

Revised Timeline



Chapter 2: Structured Literature Review

1. Introduction

Xenobiotic compounds can be described as potentially harmful chemical substances that are foreign to the human bodywhich includestherapeutic drugs, drugs of abuse and any toxic compounds (Polettini, 2006). Clinical and forensic toxicology are two distinct disciplines in analytical toxicology that use similar techniques for the detection and quantification of xenobiotics in human biological matrices (Gerostamoulos& Beyer, 2010). The analytical results produced in clinical toxicology aid in prescribing the most suitable treatment of a poisoned or intoxicated patient (Gerostamoulos& Beyer, 2010). Whilst in forensic toxicology, the results can lead to the determination of possible impairment or behavioural changes in an individual, as well as shedding light on the contribution of drugs or poisons to death in a medico-legal enquiry (Gerostamoulos& Beyer, 2010). It can thus be comprehended that a rugged technique of high discriminating power is imperative to draw the above mentioned conclusions with confidence. It should be noted that quantitative toxicological testing to aid in forensic investigations in South Africa is extremely scarce and there exist a need for the development of such services.

There are various analytical techniques for determination of unknown compounds in biological matrices such as blood and urine. Liquid chromatography tandem mass spectrometry (LC-MS/MS) detection is a current trend accepted for both the screening and quantification of compounds. A number of different parameters of LC-MS/MS can be fine-tuned for the optimal determination of the analytes of interest. This includes the initial sample preparation step, the analytical column and mobile phase composition in liquid chromatography as well as the type of mass spectrometric detection. Moreover, LC-MS/MS methods that have a multi-analyte approach are of great immediate interest in clinical and forensic toxicology due to its high throughput capability.

Analytes of interest for the development of a quantification LC-MS/MS method for this research project includes those from the benzodiazepine and antidepressant drug classes. The target analytes are 8 benzodiazepines: alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, tenazepam; 2 tricyclic antidepressants: amitriptyline and nortriptyline; and the selective serotonin re-uptake inhibitor (SSR) antidepressant, citalopram. Benzodiazepines and antidepressants are both widely

prescribed and thus commonly encountered in clinical and forensic toxicology. These types of drugs mainly work in the human brain and thus create a high risk for potential abuse of these drugs along with concerning side-effects that could lead to the causation of serious crimes.

This review will provide a concise outline of the typical strategy used by most laboratories for the identification and quantification of unknown compounds from biological matrices as well as the advantages of using liquid chromatography tandem mass spectrometry (LC-MS/MS) as a detection mechanism. The beneficial decision of including multiple analytes in the LC-MS/MS quantification method is discussed along with the need to focus on benzodiazepines and antidepressants as target drug classes. Furthermore, recently published multi-analyte LC-MS/MS methods are reviewed, focusing on the sample preparation, liquid chromatography and mass spectrometric detection aspects in order to establish some developed methods that have found to be successful.

Literature was searched for on Google Scholar as well as PubMed, ScienceDirect and Scopus electronic databases. Keywords used to make inquiries included: clinical and forensic toxicology; LC-MS/MS detection and quantification; multi-analyte procedures; benzodiazepines; antidepressants; blood, serum and urine; sample preparation; method validation. Publications were chosen between the years 1997 – 2014.

2. Summary of Analytical Process

Screening and Identification of Xenobiotics

Compounds to be determined in clinical and forensic toxicology are most of the time unknown and must be identified before quantification. There are two high-throughput methods used for the identification of unknown compounds, namely general unknown screening (GUS) and systematic toxicological analysis (STA). These procedures are comprised of an analytical method or a combination of analytical methods to simultaneously screen for a wide range of relevant xenobiotics that may be present in biological fluids in cases of unknown poisoning or intoxication (Polettini, 2006; Maurer, 2005b). The first method, STA, involves screening of comprehensive catalogues of toxicological relevant compounds whilst GUS diffidently detects and identifies all exogenous

compounds in a sample without any pre-selection (Marquet, 2012). Figure 1 depicted below displays an analytical strategy that is commonly followed for the determination of unknown compounds. The figure adapted from Maurer (2005b) will be discussed in further detail in the text below.

Strategy for determination of unknown compounds

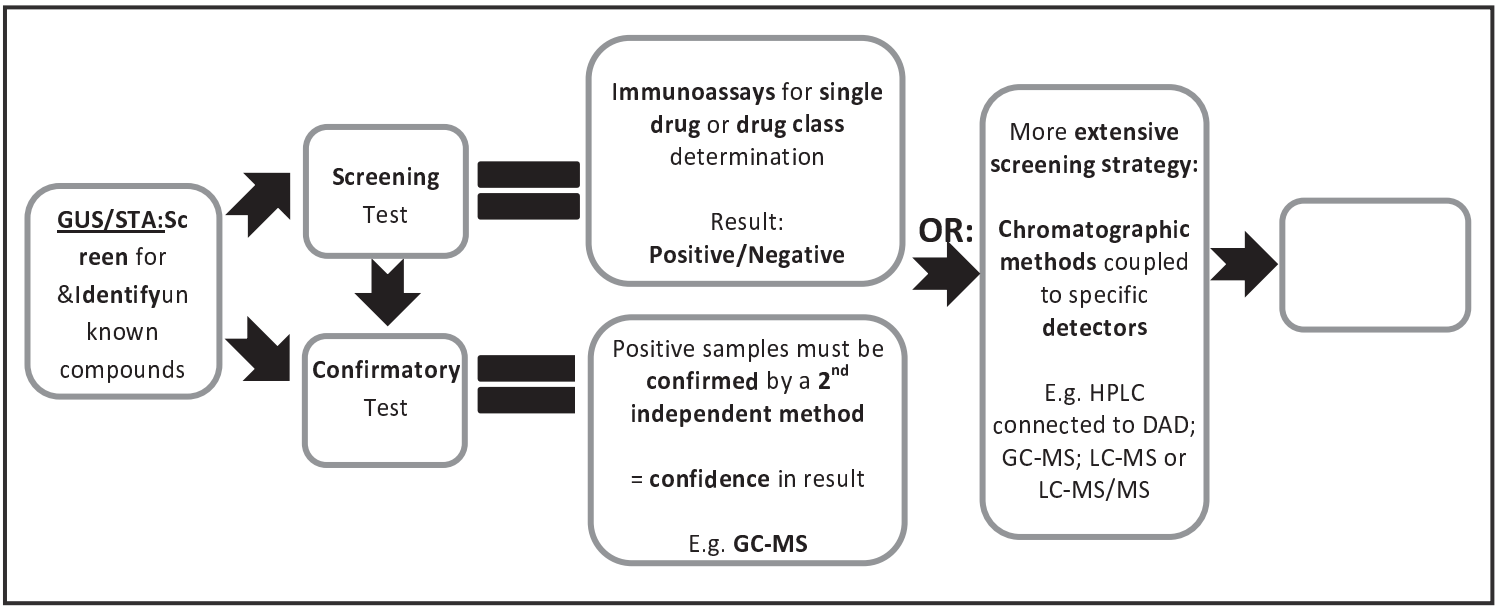


Figure 1: A flow diagram of a common analytical strategy for the detection and quantification of unknown compounds from biological matrices (Adapted from Maurer, 2005b). DAD – diode array detector; GC-MS – gas chromatography-mass spectrometry; GUS – general unknown screening; HPLC – high performance liquid chromatography; LC-MS – liquid chromatography-mass spectrometry; LC-MS/MS – liquid chromatography tandem mass spectrometry; STA – systematic toxicological analysis

Screening via Immunochemical methods

The preliminary screening of samples typically involves immunoassay techniques as it provides a prompt binary positive or negative result to indicate the presence or absence of a target analyte above a specified threshold (Sturm, 2005). A shortcoming of immunoassay-based drug testing is however that it is subject to false-positives. The reason for this is that the technique has a reduced drug sensitivity and specificity due to a high degree of cross-reactivity that can exist for each species within a class of drugs (Eichhorst*et al.*, 2012). Results could thus show the presence of the metabolites of a drug, but the detection of

exactly which form of that drug is present, may be inadequate. It is therefore imperative to perform a second confirmatory test when a positive result for one or more compound classes or target substances is found.

Confirmation

The second independent confirmatory method must provide the highest level of confidence in the result and should thus be more sensitive than the initial screening method (Maurer, 2005b). Gas chromatography - mass spectrometry (GC-MS) has long been a universal method of choice for confirmation due to its specificity, sensitivity and the availability of vast libraries of standardised spectra (Polettini, 2006). This two-step strategy comprising of a screening test followed by a confirmatory test is typically employed for the most common drugs which are usually scheduled by law and for which immunoassay kits are commercially available for (Maurer, 2005b). Cases involving the detection of drugs that do not fall under the major categories of drugs of abuse require a more all-embracing tactic.

A better strategy

A more extensive GUS or STA procedure involves the use of chromatographic techniques which are coupled to specific detectors. This pairing of devices allows for the desired identification of a much larger number of relevant compounds in an unambiguous manner. Examples of these include gas or liquid chromatography linked to diode array detectors or mass spectrometers.

Gas chromatography coupled with mass spectrometry or high performance liquid chromatography (HPLC) with diode array detection (DAD) was primarily employed by most laboratories for this purpose (Sturm, 2005). These methods can however not adequately recognise all possible toxic analytes. Gas chromatography can strictly be used for the analysis of volatile, nonpolar and thermally stable compounds of lower molecular weight. For other compounds, GC-MS requires laborious and time-consuming compound derivatization(Lee, 2013). Furthermore in cases where certain polar compounds have limited ultra violet (UV) absorbance for instance, neither GC-MS nor HPLC-DAD would be sufficient for detection (Sturm, 2005). An alternative methodology that can thus be used is liquid chromatography – mass spectrometry (LC-MS) or liquid chromatography tandem mass spectrometry (LC-MS/MS).

LC-MS and LC-MS/MS – most robust

Liquid chromatography coupled with a single stage or in particular tandem mass spectrometer have in recent years become the most robust analytical tool for the determination of drugs and their metabolites in various biological samples (Roškar&Lušin, 2012). There are many advantages, such that it allows for a wider range of compounds to be compliant with mass spectrometric detection. Unlike GC-MS, aqueous samples, hydrophilic, thermolabile and non-volatile analytes can be analysed with this technique without any derivatizationsteps (Peters, 2011). It also allows for the study of low molecular weight (<100Da) to high molecular weight (>100 000Da) drugs and metabolites (Ardrey, 2003). Another advantage of LC-MS is the use of short columns and gradient elution to give short analysis times and a reduced eluent consumption (Flanagan *et al.*, 2007). The LC-MS interface, together with a proper preparation technique, is effective in detecting analytes at concentrations that are difficult to analyse with commonly used GC-MS based techniques (Saito *et al.*, 2011; Di Rago*et al.*, 2011). LC-MS/MS has thus helped to close the gap with respect to analytes which were not sufficiently covered by the established gold standard technique GC-MS (Peters, 2011). It is clear that LC-MS/MS is highly convenient for the simultaneous analysis of an especially wide variety of drugs which makes it ideal for both screening as well as consequent quantification of compounds that follows (Maurer, 2005a).

Quantification by a Validated Method

In the usual course of events, after establishing which drugs are present, an LC-MS/MS quantification method specific to the identified compounds is then employed to determine their concentration. This is done since knowledge of the analyte concentrations ultimately allows for interpretation of forensic and clinical toxicology cases. As with most analytical techniques, LC-MS/MS quantification encompasses a standard curve approach. This means the sample under investigation and standard solutions containing known amounts of the target analyte(s) are measured under equal experimental conditions (Ardrey, 2003). The intensity of response observed for analyte(s) present in the sample is then compared to that of known amounts of the analyte(s) in standards (Ardrey, 2003).

An internal standard should typically be used when mass spectrometric quantification is performed.This substance can then be used for calibration by plotting the ratio of the

analyte signal to the internal standard signal as a function of the analyte concentration of the standards. This is done to correct for the loss of analyte during sample preparation or sample inlet. The internal standard is a compound that is very similar, but not identical to the chemical species of interest in the samples, as the effects of sample preparation should, relative to the amount of each species, be the same for the signal from the internal standard as for the signal(s) from the species of interest in the ideal case.

A suitable internal standard would control of variabilitiesor loss of compound that may arise during sample extraction, liquid chromatography injection and ionization within the mass spectrometer. The most optimal internal standard would be an isotopically labelled version of the target molecule to be quantified as it will have a similar extraction recovery, ionization response in ESI mass spectrometry, and a similar chromatographic retention time. It requires special synthesis of isotopically labelled internal standards which is expensive and time consuming. Alternatively compound analogs can be used as internal standards which are more likely available. Such internal standards will be similar to the compound to be quantified and more importantly will be slightly different by parent mass. Once the method has been developed and optimised, it needs to be validated.

Method validation can be described as the process whereby the performance characteristics of an analytical method are ascertained via laboratory studies to meet the requirements for its intended analytical applications (SANAS TG 41-01:2008). The validation process aids in the production of reliable results which is essential for drawing the correct conclusions in clinical and forensic toxicology (Peters & Maurer, 1998). Validation of an analytical method is therefore of the utmost importance for a laboratory to be able to confidently employ the method for regular testing.

There exist international recommendations for the extent of validation experiments and the acceptance criteria for validation parameters which should ideally be followed (Peters & Maurer, 1998). The South African National Accreditation System (SANAS), the United States Food and Drug Administration (FDA), the European Medicines Agency (EMEA) and the Scientific Working Group for Forensic Toxicology (SWGTOX) to name a few, have all published standard practices for method validation (SANAS TG 41-01:2008; FDA Guidance for Industry, Bioanalytical Method Validation, 2001; EMEA/CHMP/EWP/192217/2009;

SWGTOX Doc 003). Guidelines such as these have been created with the aim of preventing disparity in the validation of different bioanalytical methods by different laboratories.

Validation parameters that are widely agreed on to be tested include: selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, accuracy, and precision, different types of stability tests and the applicability (Maurer, 2005a). The validation of LC-MS/MS procedures furthermore requires the consideration of certain features apart from the typical recommendations suggested for bio-analytical method validation. Possible matrix effects (MEs) which include ion suppression or ion enhancement for example, is particularly unique to the LC-MS/MS technique in the electrospray ionization (ESI) mode and should be investigated during validation (Polettini, 2006). Stability is another important validation parameter to focus on for an LC-MS/MS technique that is to be used in forensic and/or clinical toxicology (Polettini, 2006).

Once the validation study is approved by the quality assurance department of the laboratory in which the procedure is to be performed, a standard operating procedure (SOP) is composed. A SOP describes how the procedure should be performed in detail and it is very important that the forensic analyst always follows the particulars of this document. This is to ensure run-to-run precision and it ultimately aids in a convincing testimony should forensic results be contested in court. As soon as analytes for which the quantification method has been designed for are found to be present in a test sample via routine screening, the LC-MS/MS method can then be pursued for quantification purposes.

Before a quantification method is initially developed, it needs to be decided which analytes will be targeted. Seeking to include a number of different analytes in the quantification method, instead of just focusing a single analyte, will have many advantages as explained in the following section.

3. Method Focus

Multi-analyte approach

It is sought-after in forensic toxicology to develop procedures that allow for the concurrent analysis of multiple relevant drugs within a certain drug class or of numerous drug classes that are closely related and/or commonly abused (Peters, 2011). This is due to the

conservation of both time and resources that such multi-analyte procedures can sanction during method development and validation (Peters, 2011). Compared to single-analyte approaches, it is practically and economically much more feasible and the analytical strategy is simplified.

To further elaborate, combining a number of analytes in a single method allows for streamlining of routine practices within the laboratory and a decreased specimen volume can be used for such analysis. Analytes of different drug classes are monitored in a single matrix with a single sample preparation and analysing method (Remane*et al.*, 2010). This is of particular importance in forensic toxicology where only a limited amount of sample may be available and in addition it is not initially known how many analyses would have to be performed on that sample (Remane*et al.*, 2010). Furthermore it results in a smaller number of methods to be created by the laboratory to cover a broad spectrum of analytes (Peters, 2011).

There have been a number of recent publications that report the successful simultaneous analysis of a very large number of analytes in various biological matrices by the use of LC-MS/MS (Di Rago*et al.*, 2014; Hegstad*et al.*, 2014; Montenarh*et al.,* 2014a, b, c; Bjørk*et al.*, 2013; Lee, 2013; Johnson-Davis e*t al.*, 2012; Sauve*et al.*, 2012; Verplaeste*et al.*, 2012; Remane*et al.*, 2011, 2010; De Castro *et al.*, 2007). Di Rago*et al.* (2014) presented a LC-MS/MS method that has proven to be applicable for the quantification of 132 acidic and neutral analytes in whole blood. A rapid protein precipitation extraction technique using acetonitrile and a small sample volume of 100µl of blood was fashioned by this group as there was a need for quicker turnaround times. This sample preparation along with automated data processing resulted in a technique to cope with large numbers of samples and it ultimately enhanced the laboratory's performance. Lee (2013) published a robust method for the concurrent screening of over 170 drugs of abuse and drugs commonly found in emergency cases in urine samples via LC-MS/MS. The method was proven to be robust via validation experiments using system suitability mixture and quality control materials. The study targeted drugs that are most prevalent and consequently the screening method was successfully applied to almost 500 urine samples that was collected over time from intoxicated patients at an emergency centre, proving its applicability.

Some multi-analyte procedures for screening allow consecutive quantification such as that devised by Kratzch*et al.* (2004). This group for instance developed and fully validated a single stage LC-MS procedure to quantify benzodiazepines in the selected-ion monitoring (SIM) mode once the analytes have first been recognized to be present in serum samples by means of screening in the scan mode using the authors' LC-MS library (Kratzch*et al.*, 2004). The same extracts that were separated via their usual liquid chromatography procedure used for screening, are then applied in the quantification assay that functions in the positive atmospheric pressure chemical ionization (APCI) SIM mode. Remane*et al.* (2011) as another example could do a full validation of a LC-MS/MS procedure for both the targeted screening and quantification of 34 antidepressants in plasma as part of a comprehensive multi-analyte approach.

Marquet (2012) states that there exist numerous targeted screening methods that involve single-stage mass spectrometry in the SIM mode or tandem mass spectrometry in the selected reaction monitoring (SRM) mode for the analysis of almost all toxic compounds and drug classes and that these methods also permit the quantification of the targeted compounds. For the purpose of this review, LC-MS/MS methods that target benzodiazepines (e.g. Kratzch*et al.*, 2004 above) and antidepressants (e.g. Remane*et al.*, 2011 also mentioned above) will be a focus.

Target analytes: Benzodiazepines and Antidepressants

Multi-analyte methods can cover one or more drug classes, with each class exhibiting unique chemical properties based on the chemical structure of the compounds within that class. An example of an analytical method featuring a single drug class is that for the detection and/or quantification of nine clinically significant tricyclic antidepressants (TCAs) in human plasma or serum (Johnson-Davis *et* al*.,* 2012). This method involves ultra pressure liquid chromatography-tandem mass spectrometry which aids in therapeutic drug management specifically of tricyclic antidepressants. Conversely Bjørk*et al*. (2013) published a LC-MS/MS method incorporating for instance as many as four different drug classes, including opioids, cocaine, amphetamines and benzodiazepines to quantify a total of 31 significant illicit and medicinal drugs and metabolites.

Methods are mostly designed to target analytes based on their pharmacological effects and urgency for screening and quantification such as classic drugs of abuse, therapeutic drugs with abuse potential or relevant compounds in the context of driving under the influence of drugs as described by Remane, *et al.* (2010). Benzodiazepines and some antidepressant drugs are among some of the most frequently encountered analytes identified in biological matrices during routine toxicological analysis. These prescription drug classes have been found to be in close association with drug dependency amongst patients owing to some of the satisfying effects they induce, consequently making them popular amongst abusers (White &Taverner, 1997).

Benzodiazepines

Benzodiazepines are frequently misused since it heightens the affinity of $GABA_A$ receptors in the nervous systems for its endogenous ligand, the neurotransmitter gamma-aminobutyric acid (GABA)leading to sedative and anti-anxiety effects (Page *et al.*, 2002). The amnestic properties of benzodiazepines make them desirable agents for use in a drug-facilitated sexual assault, therefore making this drug class an important forensic toxicological focus (Morris-Kukoski, Schaff, &Reda, 2012). In addition, psychomotor and cognitive function is badly influenced and behavioural changes such as a loss of inhibition in the abuser can be observed. It has also been found that high doses of benzodiazepines could cause amplified aggression in certain susceptible individuals (Drummer, 2009).

Figure 2 below contains the structures of the eight benzodiazepines that are routinely tested and it can be seen that the main chemical structure is common to most of the molecules and that they only differ in the presence of varying substitutes.

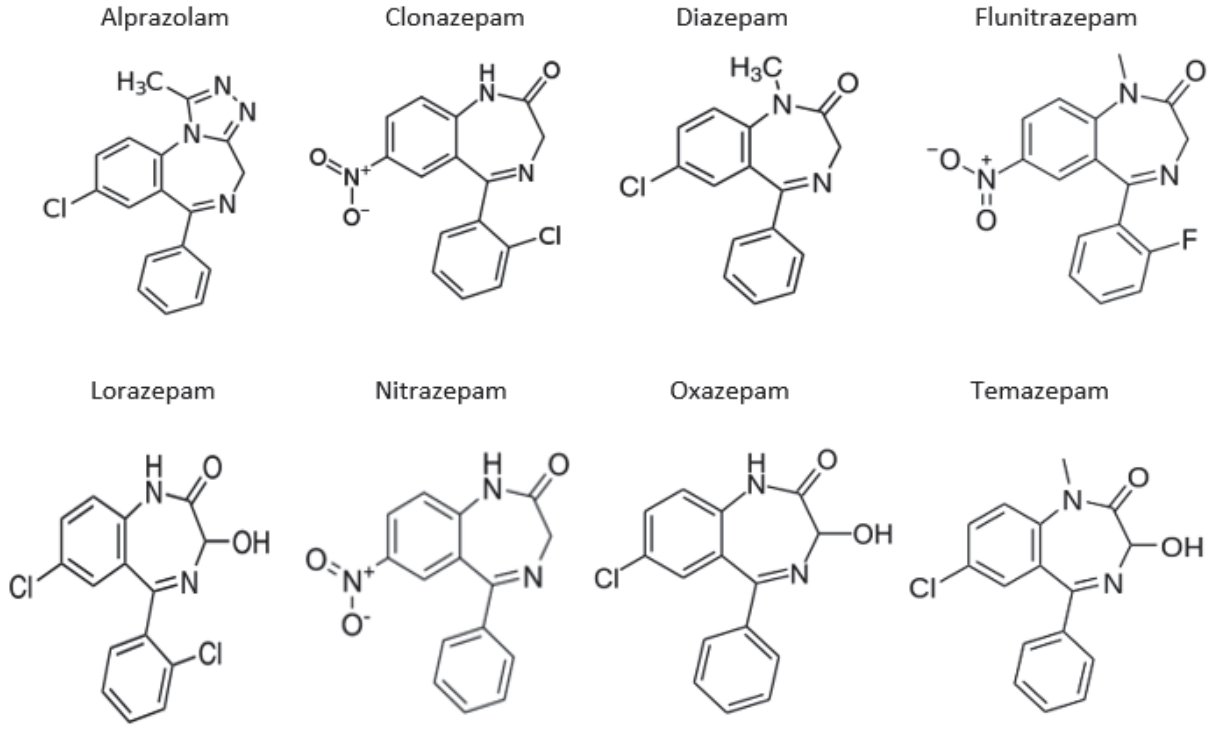


Figure 2: Structure of 8 routinely tested benzodiazepines (Salomone*et al.*, 2011)

Antidepressants

Antidepressants comprise a diverse group of compounds including the traditional tricyclic antidepressants (TCAs) and the newer generation of antidepressants, the selective serotonin re-uptake inhibitors (SSRIs). These antidepressants are also abused. Tricyclic antidepressants have adverse side-effects such as an increase in suicidal thoughts and too high doses can cause cardiac disturbances, respiratory depression, metabolic acidosis, convulsions and coma (Jickells&Negrusz, 2008). SSRIs, including citalopram, are preferred prescription antidepressants since they are less toxic. Serotonin syndrome(shivering, diarrhea, confusion, severe muscle tightness, fever, seizures, and death)and other risks such as cardiac conductive failures and rhabdomyolysis that are associated with the use of SSRIs are however taken too lightly (Jickells&Negrusz, 2008).

Figure 3 illustrates the core three-ring structures of two tricyclic antidepressants namely amitriptyline and nortriptyline.Nortriptyline is also an active metabolite of the TCA amitriptyline (Johnson-Davis *et al.,* 2012). The structure of citalopram, a common antidepressant from the SSRI group, is also depicted in figure 3.

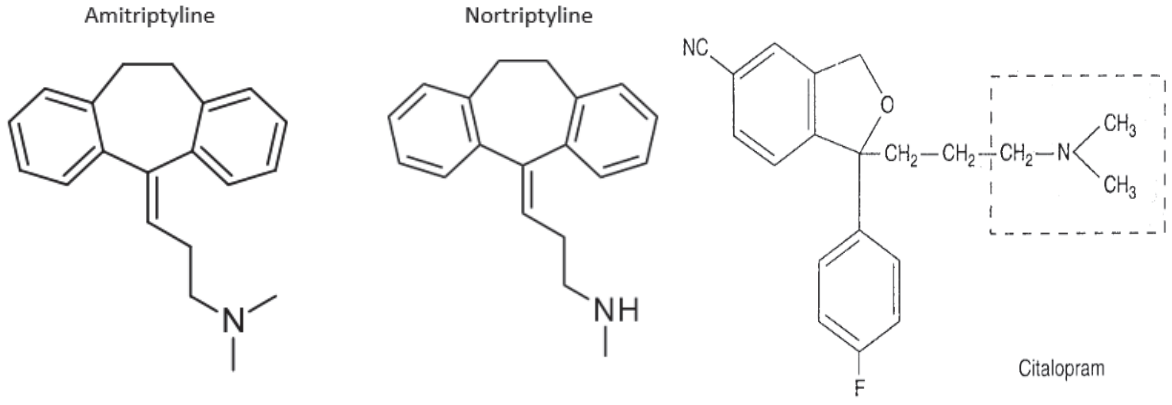


Figure 3: Structures of two tricyclic antidepressants, amitriptyline and nortriptyline, (Johnson-Davis *et* al*.,* 2012) and that of citalopram, a selective serotonin reuptake inhibitor (Larsen, 2000)

Certain sedatives and antidepressants as described above have been implicated in impaired driving cases (Jickells&Negrusz, 2008). Two special issues of Forensic Science Reviews (vol. 14, 2002, and vol. 15, 2003) have been published to show that driving is indeed affected by many drugs, including opioids and benzodiazepines (Jickells&Negrusz, 2008). This was demonstrated through the use of driving simulators, in on-road driving studies as well as from anecdotal and epidemiological reports (Jickells&Negrusz, 2008). It is clear that driving under the influence of drugs (DUID) cases are not solely associated with only illegal drugs. Moreover, an increased intensity of driving impairment is caused by the use of alcohol in combination with recreational or prescription drugs (Jickells&Negrusz, 2008).

Concentration ranges of these drugs

Schulz *et al*. (2012) spent over 20 years to abstract data from different sources to compile a comprehensive list of reported therapeutic and toxic plasma concentration ranges of a vast number of xenobiotics. The aim is to provide instant information for the assessment of the significance of drug levels in clinical and forensic toxicology. Table 1 indicates the plasma levels in man that coincide with the therapeutic, toxic and comatose or fatal concentration ranges that have been affirmed by Schulz *et al*. (2012) of the above cited benzodiazepines and antidepressants. The table shows evidence that underlines that the toxicity of these drugs can be life threatening.

Table 1: A table containing therapeutic, toxic, and comatose-fatal human blood-plasma concentrations in ng/mL of some common benzodiazepines and antidepressants (Schulz e*tal.*, 2012)

Drug Class		Compound	Blood-plasma concentration (ng/mL)		
			Therapeutic	Toxic	Comatose-fatal
Benzodiazepines		Alprazolam	5 - 50/80	100 - 400	
		Clonazepam	50 - 110	220	5000 - 6000
		Diazepam	100 – 200/250	3000 - 5000	
		Flunitrazepam	5 - 15	50	
		Lorazepam	20/80 - 250	300 - 500	
		Nitrazepam	30 - 100	200 - 300	5000
		Oxazepam	200 - 1500	2000	3000 - 5000
		Temazepam	20 – 150/900	1000	8200; 14000
Antidepressants	Tricyclic	Amitriptyline	50 - 300	500 - 600	1500 - 2000
		Nortriptyline	20 - 200	300; 500	1000 - 3000
	SSRI	Citalopram	50 - 110	220	5000 - 6000

ng/mL – nanogram per millilitre; SSRI – Selective serotonin re-uptake inhibitors

It is clear that abuse of these types of drugs is not without risk: other than the cause of driving impairment which can have its own severe consequences, abuse can lead to irreversible damage to a person's system and moreover fatal intoxications have been reported (Schulz e*tal.*, 2012). It is also important to clarify if a crime was committed by a person who was under the influence of drugs. For these reasons screening procedures are needed in both forensic and clinical toxicology to allow for the reliable determination of substances in biological matrices.

The Sample Matrix

The choice of specimen to analyse is regularly influenced by the forensic or clinical situation (Vindenes*et al.*, 2012). Blood, serum, plasma and urine are the typical specimens for toxicological analysis. For the purpose of this review, blood matrices and urine will be given attention to. There are a number of other less conventional biological specimens that have also been collected and analysed for toxicological purposes which include: dried blood spots, hair, oral fluid (saliva), umbilical cord, placenta, meconium, tissue (such as brain and adipose tissue), sweat, breath, nail clippings, cerebrospinal fluid and even fingerprints (Saito *et al.*, 2011; Nakamura, 2011).

Blood Matrices

Whole blood, often interchangeable with plasma or serum in most methods, is the most analysed type of specimen in forensic toxicology and this is especially due to its ease of collection from deceased or unconscious individuals (Sturm, 2005). It is the sample of choice in cases where toxic effects, committing a crime, or driving under the influence of drugs needs to be assessed (Montenarh*et al.*, 2014a). The reason for this is that the drug concentration levels in blood correlate best with actual pharmacological effects (Flanagan *et al.*, 2007; Maurer, 2005a). Therefore blood matrices are especially preferred when quantitative analysis is required to interpret if a therapeutic or toxic concentration was present at the time of the offence (Montenarh*et al.*, 2014a).

Comprehensive lists with therapeutic, toxic and fatal drug concentrations can then be utilised by pathologists in routine casework to grasp already determined concentration levels and hence to assess if intoxication was a likely cause of death etc. (Reis *et al.*, 2007). There exist a number of such resources such as that published by Schulz *et al.* (2012) mentioned above and also The International Association of Forensic Toxicologists (TIAF) reference blood levels of therapeutic and toxic substances, to name a few. According to Linette (2012) there are a number of factors that however need to be considered when interpreting post-mortem measurements in relation to published *in vivo* plasma or serum drug levels under therapeutic circumstances. Some of these factors for instance include the analytical methodology of measurement, drug tolerance development of the individual and

post-mortem drug redistribution (Linette, 2012). Pathologists should thus be critical in the interpretation of the toxicological findings.

Urine

Urine is the most frequent specimen used in clinical situations. It is easier and less invasive to obtain a large volume of urine compared to a blood sample from a patient that has damaged veins and additionally human urine imposes less of a health risk to laboratory staff (Flanagan *et al.*, 2007). The value of urine samples is that drug and drug metabolite concentrations tend to be much higher than in blood and it is thus a well-established matrix for screening analysis (Montenarh et al., 2014a). The detection window is longer in urine than in blood (Lee, 2013) and sometimes drugs are found to be present in urine but not in a blood sample from the same person (Montenarhet al., 2014a). It must be remarked that certain drug classes, for example benzodiazepines, are considerably metabolized before it is excreted into urine. In such cases blood plasma should be the specimen of choice for the detection of the parent compound (Flanagan *et al.*, 2007). It is more difficult to link urine concentrations to pharmacologic effects

4. Overview of multi-analyte LC-MS/MS procedures

Method Development

Development of an LC-MS/MS method generally requires the development of three separate techniques (Waters, n.d.):

- Sample preparation
- Liquid Chromatography (LC) Separation
- Multiple Reaction Monitoring (MRM) Mass Spectrometry

The LC-MS/MS method as a whole is optimised in an iterative manner as modifications in one of these methodologies can affect another. The MRM mass spectrometry method is reasonably the first method to be established as it indicates that the target compounds can indeed be detected by mass spectrometry (Waters, n.d.). This is performed using a solution of commercially bought reference standards of the analytes of interest which is of high purity. Additionally, the MRM method can be fine-tuned so that possible spurious mass transitions are at a minimum (Waters, n.d.). The mass spectrometry method subsequently

aids in the optimization of the LC method as it verifies that the analytes can be separated from one another and from possible other redundant compounds (Waters, n.d.). The LC method is also successively needed to establish the cleanliness and efficacy of the sample preparation technique. Ultimately the LC-MS/MS method is tested by introducing the drug-free biological matrix (be it blood, serum, urine etc.) spiked with the reference standards in order to investigate any effects of the components of the matrix other than the analytes of interest. Upon this step further modifications are made with the aim of obtaining the final method which is then to be validated.

The following tables summarises a selection of recently published LC-MS/MS multi-analyte methods. Table 2 contains LC-MS/MS methods targeting a number of different drug classes, whereas table 3 and 4 looks at methods targeting solely benzodiazepines and antidepressants respectively. Basic information about the sample matrix tested, sample preparation, liquid chromatography column, mobile phase, ionization type, mass spectral detection mode as well as validation data for each procedure is reviewed. Thereafter a discussion about the three methodologies namely sample preparation, liquid chromatography separation and mass spectrometric detection, which make up a LC-MS/MS method, will follow.

Table 2: Summary of a selection of recently published LC-MS/MS multi-analyte methods

Target Analytes	Matrix	Sample Preparation	LC		MS		Validation Data	Reference
			Column	Mobile Phase	Interface	Mode		
130 Analytes including benzodiazepines, Z-drugs, antidepressants, neuroleptics, opioids, new synthetic drugs, and phosphodiesterase type 5 inhibitors	Whole blood, plasma, serum, post-mortem blood, liver tissue, gastric content, hair and urine	Different LLE methods	Shimadzu Prominence LC 20 AC system		AB SCIEX 3200 Q-TRAP® linear ion-trap quadrupole mass spectrometer		LOD, selectivity, recovery, matrix effects, process efficiency, precision, applicability	Montenarh *et al.* (2014a)
			Waters SunFire C18 column (2.1 x 150 mm, 3.5 µm)	Gradient elution. 10 mM ammonium formate in water plus 0.1% formic acid, pH 3.4: acetonitrile plus 0.1% formic acid	ESI^+, MS/MS	MRM		
132 Acidic and neutral drugs and poisons	Whole blood	Protein Precipitation with acetonitrile	Shimadzu 20AD HPLC		ABSciex API 2000™ mass spectrometer equipped with a linear accelerator collision cell quadrupole mass spectrometer		Selectivity, linearity, accuracy, precision, stabilities, LOQ, LOD, matrix effects, recovery, applicability	Di Rago*et al*. (2014)
			Kinetex XB-C18 (4.6 x 150 mm, 5 µm)	Gradient elution. 25 mM ammonium acetate in water, pH 7.5: acetonitrile	ESI^+ and ESI^-, MS/MS	MRM		

35 Different analytes including cannabis, pregabalin, opioids, central nervous system stimulants, benzodiazepines and related agents	Urine	Dilution	Waters Acquity UPLC I-Class FTN system		Xevo TQ-S tandem-quadrupole mass spectrometer equipped with a Z-spray electrospray interface		Linearity, LOD, LOQ, precision, bias, matrix effects, specificity, carry-over, stability, applicability	Hegstad*et al*. (2014)
			Acquity HSS T3-column (2.1 x 100 mm, 1.8 µm)	Gradient elution. 0.1% Formic acid in water: methanol and 0.1% Formic acid in water: acetonitrile.	ESI^+ and ESI^-, MS/MS	MRM		
19 Drugs of abuse and metabolites including opioids, cocaine, amphetamines and benzodiazepines	Whole blood	Automated SPE	Agilent 1100 series HPLC system		Waters Quattro micro tandem quadrupole mass spectrometer		Specificity, matrix effects, recovery, linearity, LOD, LOQ, precision, accuracy, applicability	Bjørk*et al*. (2013)
			Varian Pursuit 3 C18 column (100 x3 mm, 3 µm)	Gradient elution. 2 mM Ammonium formate buffer/8% acetonitrile, pH 5.3: 100% methanol.	ESI^+, MS/MS	MRM		
136 Analytes including antidepressants, neuroleptics, benzodiazepines, beta-blockers, oral antidiabetics, and analytes relevant in the context of brain death diagnosis	Plasma	LLE	TF Accela UHPLC system		TSQ Quantum Access mass spectrometer		Recovery, matrix effects, process efficiencies	Remane*et al*. (2010)
			TF Hypersi GOLD Phenyl column (100 x 2.1 mm, 1.9 µm)	Gradient elution. 10mM ammonium formate in water plus 0.1% formic acid, pH 3.4: acetonitrile plus 0.1% formic acid.	$APCI^+$, MS/MS	MRM		

Table 3: Summary of a selection of recently published LC-MS/MS targeting benzodiazepines

Target Analytes	Matrix	Sample Preparation	LC		MS		Validation Data	Reference
			Column	Mobile Phase	Interface	Mode		
19 Anlaytes including benzodiazepines and Z-drugs	Whole blood, plasma and serum	LLE	Shimadzu Prominence LC 20 AC system		AB SCIEX 3200 Q-TRAP® linear ion-trap quadrupole MS		Selectivity, matrix effects, recovery, process efficiencies, accuracy, precision, stabilities, Lod, loq	Montenarh *et al.* (2014b)
			Waters SunFire C18 column (2.1 x 150 mm, 3.5µm)	Gradient elution. 10 mM ammonium formate in water plus 0.1% formic acid, pH 3.4: acetonitrile plus 0.1% formic acid	ESI^+, MS/MS	MRM		
11 Benzodiazepines	Ante-mortem and post-mortem blood	LLE	Waters Acquity UPLC module		Waters Quattro Premier XE tandem mass spectrometer		Linearity, precision, accuracy, LOQ, LOD, recovery, specificity, carry-over, matrix effects, stability	Sauve*et al.* (2012)
			BEH C18 column (2.1 x 100 mm, 1.7µm)	Gradient elution. Acetonitrile: 5mM ammonium acetate buffer, pH 5.0.	ESI^+, MS/MS	MRM		
Benzodiazepines, benzodiazepine-like hypnotics and some metabolites	Urine and whole blood	SPE	UPLC Shimadzu system		AB SCIEX 3200 Q-TRAP® MS		Selectivity, matrix effects, recovery, linearity, LOD, LOQ, precision, accuracy	Verplaetse *et al.* (2012)
			AcquityBEH C18 column (2.1 x 50 mm, 1.7µm)	Gradient elution. 10 mM Ammonium bicarbonate in water, pH 9: methanol.	ESI	sMRM		

Table 4: Summary of a selection of recently published LC-MS/MS targeting certain antidepressants

Target Analytes	Matrix	Sample Preparation	LC		MS		Validation Data	Reference
			Column	Mobile Phase	Interface	Mode		
33 Antidepressants	Whole blood, plasma and serum	LLE	Shimadzu Prominence LC 20 AC system		AB SCIEX 3200 TRAP® linear ion-trap quadrupole mass spectrometer		Selectivity, matrix effects, recovery, process efficiency, accuracy, precision, stabilities, limits	Montenarh *et al.* (2014c)
			Waters SunFire C18 column (2.1 x 150 mm, 3.5 µm)	Gradient elution. 10mM ammonium formate plus 0.1% formic acid, pH 3.4: acetonitrile plus 0.1% formic acid.	ESI^+, MS/MS	MRM		
9 Antidepressants	Serum or plasma	Rapid Protein Precipitation with 50:50 MeOH: acetonitrile	Waters Acquity UPLC		Tandem quadrupole detector		No validation data	Johnson-Davis *et al.* (2012)
			Acquity UPLC HSS T3 column (2.1 x 50 mm, 1.8 µm)	Gradient elution. 0.1% Formic acid in water: 0.1% formic acid in acetonitrile.	ESI^+, MS/MS	MRM		
34 Antidepressants	Plasma	LLE	TF Accela UHPLC system		TSQ Quantum Access mass spectrometer		Selectivity, cross talk, recovery, matrix effects, process efficiencies, linearity, accuracy,	Remane*et al.* (2011)
			TF Hypersil GOLD Phenyl column (100 x 2.1 mm, 1.9 µm)	Gradient elution. 10mM ammonium formate in water plus	$APCI^+$, MS/MS	MRM		

				0.1% formic acid, pH3.4: acetonitrile plus 0.1% formic acid.			precision, stabilities, LOQ, LOD, applicability	
14 Antidepressants and their metabolites	Plasma	On-line SPE	Symbiosis Pharma System		Waters Quattro Premier tandem spectrometer		Linearity, LOQ, LOD, precision, accuracy, selectivity, specificity, stability, matrix effects, recovery	De Castro *et al*.(2007)
			Gemini C18 guard column (4 x 2.0 mm, 5 µm)	Gradient elution. 10mM Ammonium hydrogencarbonate, pH 10: acetonitrile	ESI+, MS/MS	MRM		

APCI - atmospheric pressure chemical ionization; ESI - electrospray ionization;HPLC - high performance liquid chromatography; LC - liquid chromatography; MS - mass spectrometry; LLE - liquid liquid extraction; LOD - limit of detection; LOQ - limit of quantification;MeOH - methanol;µm - micrometer; mm - millimeter; MRM - multiple reaction monitoring;mM – milimolar; MS/MS - tandem mass spectrometry; SPE - solid-phase extraction; UPLC - ultra-performance liquid chromatography;

Sample Preparation

Sample preparation for the previously mentioned biological matricesmayconsist of simple dilution, e.g. of urine samples, or involve several samplepre-treatment and extraction steps.Pre-treatment of samples by the removal of interfering compounds, that are present within the biological matrices is needed for instrument soiling and to prevent clogging of the analytical column (Roškar&Lušin, 2012). These interfering compounds typically include lipids, proteins and salts which can also affect the ionization efficiency of the mass spectrometer (Nakamura, 2011). Initial sample preparation is thus an important step to aid in achieving accurate validation results as it ultimately results in a heightened sensitivity and selectivity of the instrument. Other problems such as interfering peaks might arise with the use of spectroscopic detection (fluorescence/UV-absorbance) if samples are insufficiently pre-treated. This phenomenon is less likely to be an issue when using LC-MS/MS procedures whereby the preparation of samples can be relatively straightforward (Roškar&Lušin, 2012).

There are various types of sample work-up procedures and selection of the most appropriate technique is highly dependent on the sample size, sample matrix as well as analyte characteristics and their expected concentrations (Roškar&Lušin, 2012). Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are some of the most broadly used and well-established sample preparation techniques (Roškar&Lušin, 2012). An indication of the use of LLE and SPE can be observed in the above tables, 2, 3, 4. Some basic approaches to sample preparation are simple direct injection of the sample or protein precipitation (PP) methods (Peters, 2011). The process of PP simply involves the addition of a precipitating solvent, subsequent homogenization and centrifugation (Nakamura, 2011). As can be seen from Table 2 and 3 above, both Di Rago*et al.* (2014) and Johnson-Davis *et al.* (2012) successfully used protein precipitation as a rapid sample workup technique.

Urine contains less interfering matrix compounds, hence simple dilution for preparation of urine samples have been published previously with a dilution factor ranging from five-fold up to 50-fold (Anderssonetal., 2008; Svenssonetal., 2007; Gustavssonetal., 2007).Eichhorst*et al.* (2009) for instance performed a ten-fold dilution of urine after an extra hydrolysis step was performed to obtain adequate extraction of target analytes.

An enzymatic hydrolysis step may be needed during urine sample preparation for the cleavage of glucuronic or sulphuric acid conjugates to compounds that are more easily measurable by sensitive GC-MS (Peters, 2011; Sturm, 2005). This kind of treatment is not necessarily needed for analysis by LC-MS/MS since it can directly examine conjugated compounds (Peters, 2011).

Montenarh*et al*. (2014a) recently published a LC-MS/MS multi-analyte approach using a single sample preparation tactic in eight different biological matrices for the fast target screening and reliable identification of 130 analytes that are encountered in clinical and forensic toxicology on a regular basis. These matrices include whole blood, plasma, serum, post-mortem blood, liver tissue, gastric content, hair, and urine (Montenarh et al., 2014). Sample preparation is thus simplified as there is no need to follow a completely different extraction method for every type of biological sample to be analysed.

Liquid Chromatography Separation

There exist a wide range of possible mobile-phase properties together with the choice of numerous different kinds of stationary phases when it comes to the separation of analytes by liquid chromatography. During method development, the mobile phase composition and analytical column are some of the parameters to consider experimenting with to determine what combination of conditionsyield the best chromatography. The standard reversed-phase C_{18} analytical column is widely used in the field of LC-MS/MS (De Castro *et al*., 2007; Sauve*et al*., 2012; Verplaetse *et al*., 2012; Bjørk*et al*., 2013; Di Rago*et al*., 2014; Montenarh*et al.,* 2014c), though the phenyl-bonded silica columns are also popular (Remane*et al*., 2010; Remane*et al.,* 2011). The general means of retaining analytes in the LC column during a run are similar between different stationary phases however slight changes in their surface chemistries lead to different selective characteristics (Marques & Bramble, 2007). Along with the selection of a stationary phase for separation of compounds, the type of mobile phase also highly affects the efficiency of separation.

Mobile phase compositions such as acetic acid, formic acid, ammonium formate and ammonium acetate are commonly used in liquid chromatography for the separation of a wide variety of different compounds (Tables 2, 3 and 4). These eluents are suitable for positive electrospray ionization (as mentioned below) due to the likeliness of proton

donation (Nakamura, 2011). Other LC parameters to optimize during method development include the use of gradient elution of mobile phases and the flow rate of the system.

Mass Spectrometric Detection

The pairing of liquid chromatography to tandem mass spectrometry is a widely used analytical technique for the qualitative and quantitative analysis. Mass spectrometric detection requires the ionization of analytes whereby the two most common ionization mechanisms are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). The less soft ionization technique, ESI is more appropriate for testing polar to ionic compounds versus unionized analytes (Nakamura, 2011). Verplaeste*et al*. (2012) performed a study whereby benzodiazepine hypnotics were target analytes and found ESI to have higher ionization efficiency than APCI when these two ionization interfaces were investigated along with six different mobile phases. This was found to be especially evident with an increase in the organic eluent portion. Positive electrospray ionization is frequently used for the analysis of basic benzodiazepines and similar compounds (De Castro *et al.*, 2007; Sauve*et al.*, 2012;Bjørk*et al.*, 2013; Hegstad*et al.*, 2014;Montenarh*et al.,* 2014b). The use of more acidic mobile phases advances the production of positive ions (Nakamura, 2011). Ions that result from ionization are transferred within the mass spectrometer through a vacuum into a mass analyzer.

Mass analyzers in a mass spectrometer are quadrupoles that are composed of four shafts to which direct current (DC) and radio frequency (RF) voltages are applied to. An ion of a specific mass-to-charge ratio (m/z) will be stable and can pass through the quadrupole only when a specific DC/RF voltage combination is applied. Quadrupoles are thus also referred to as mass filters. A single quadrupole system (Q) contain only one mass filtering quadrupole whilst triple quadrupole systems (QQQ) consist of three quadrupoles where Q1 and Q3 are working as mass filters while Q2 acts as a collision cell. (Schreiber, 2010)

Single quadrupole systems are typically used in a Selected Ion Monitoring (SIM) mode by which a fixed set of DC and RF voltages is applied to the quadrupole and thus only a single m/z will pass through to be detected. Ions with other m/z are filtered out. Multiple Reaction Monitoring (MRM) is commonly used for quantitative analysis using triple quadrupole MS/MS. The first quadrupole filters a specific precursor ion of interest and ions

generated within the ion source with different m/z will not pass Q1. Q2 is optimised to produce a characteristic product ion via collision of the precursor ion with a neutral collision gas (e.g. nitrogen). Generated product ions are then transferred into the third quadrupole where only a specific m/z is allowed to pass and any other product ions are filtered out at Q3. This allows both increased sensitivity and selectivity. (Schreiber, 2010)

Single and triple quadrupole systems allow the detection of many SIM and MRM transitions respectively and consequently quantification of many targeted analytes in a single experiment is possible. Additional SIM and MRM transitions have to be detected to perform identification of quantified compounds whereby the most intense ion is referred to as the 'quantifier' and any additional ions are called 'qualifiers' which confirms the presence of the target compound. (Schreiber, 2010)

Triple quadrupole MS/MS systems provide in comparison to single quadrupole MS systems a number of advantages. This includes higher selectivity which results in less interference of co-eluting compounds and matrix; better signal-to-noise which allows a lower limit of quantification; a wider linear range of quantification is possible; MRM is more reliable for identification compared to IM and better accuracy and reproducibility is warranted in particularly at low concentrations of analytes.

5. Conclusion

Liquid chromatography-tandem mass spectrometry has shown to be an extremely useful and increasingly important technique for screening and quantification of drugs and poisons in forensic and clinical toxicology (Pitt, 2013). Compared to immunochemical techniques and gas chromatography mass spectrometry, LC-MS/MS allows for the determination of a much wider range of compounds. LC-MS/MS is thus suitable for the development of methods to analyse multiple analytes in a single run. This is advantageous due to its high-throughput capability and streamlining of processes. Development of such procedures in the forensic toxicology profession in a country such as South Africa is indispensable to cope with analytical result generation demands and where there is minimal research. Prescription drugs such as benzodiazepines and antidepressants are increasingly illicitly abused and there is a range of adverse side-effects associated with the abuse of these drugs that are of clinical and forensic importance. Analysis of these drugs in biological samples

such as blood, serum and urineto obtain their concentration levels could thus aid in investigation of crimes such as drug-facilitated assault, driving under the influence of drugs and even suicide.

Method development involves the creation of a sample preparation technique to extract the target analytes and to remove other endogenous matrix compounds. There is a number of different extraction techniques, ranging from simpler to more complex methods and the decision of which method to employ is highly motivated by its efficiency and thetime needed to perform this step. A liquid chromatography method is also developed and optimised by testing different parameters such as the analytical column and mobile phases to get the most suitable separation of compounds. Lastly, the mass spectrometric detection systemwith its ionization of compounds and multiple reaction monitoring will only monitor and detect compounds that produce a specific parent ion as set by the analyst followed by formation of a specific product ion for high sensitivity and specificity.

It is paramount to validate developed LC-MS/MS procedures to guarantee their usefulness and reliability for their envisioned purposes (Polettini, 2006). Validation parameters to especially investigate with LC-MS/MS are matrix effects and stability of analytes. Once this is fully established, the benefit of incorporating the validated techniques into routine analysis can successfully be achieved (EMEA/CHMP/EWP/192217/2009).This will aid in warranting the generation of reliable results as steadfast analytical data is crucial for the proper interpretation of toxicological findings. Erroneous results can be contested in court and furthermore it could result in unfair legal penalties for the defendant or even incorrect treatment of patients which in itself can have severe consequences (Polettini, 2006).

It is thus clear that the development and validation of multi-analyte LC-MS/MSprocedures to detect and quantify drugs is very helpful for clinical and forensic examinations and more such procedures should be developed in the field of toxicology in South Africa to experience its benefits.

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Chapter 3: Research Article

Development and Partial Validation of a Method for the Quantification of Benzodiazepines and Antidepressants in Whole Blood, Serum and Urine by Liquid Chromatography - Tandem Mass Spectrometry

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Abstract

Multi-analyte procedures are sought-after in forensic toxicology as it allows for the conservation of both time and resources. The aim of the present study was to develop a multi-analyte liquid chromatography – tandem mass spectrometry (LC-MS/MS) method for the quantification of 11 different drugs in whole blood, serum and urine. The target analytes are eight benzodiazepines: alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, temazepam; two tricyclic antidepressants: amitriptyline and nortriptyline; and the selective serotonin re-uptake inhibitor (SSR) antidepressant, citalopram. The analytes were extracted from a low sample volume of 100 µl using protein precipitation with acetonitrile and were separated with a Kinetex™ 2.6 µm C18 100 Å, LC Column (50 x 3.0 mm). Gradient elution was performed using aqueous 2 mM ammonium acetate containing 0.2% formic acid and 2mM ammonium acetate in acetonitrile containing 0.2% formic acid as the organic mobile phase. The analytes were quantified by MS/MS (AB SCIEX 3200 TRAP® linear ion-trap quadrupole mass spectrometer) using multiple reaction monitoring in positive electrospray ionization mode and two transitions per analyte. Diazepam-d_5 and dioxepin-d_3 were used as internal standards. The run time of the method was 9.5 minutes including equilibration time.Precision and accuracy validation experiments were performed for blood, serum and urine samples. Partial validation was achieved in the time available for the method in blood and sensitivity, recovery, matrix effects and stability tests were performed. Applicability of the developed method was tested by analysing five different authentic samples.

Keywords: LC-MS/MS quantification; benzodiazepines; antidepressants; method development; validation

1. Introduction

Historically drugs of abuse such as cocaine and heroin have long been considered to be high-risk drugs with many adverse side-effects. It should however be acknowledged that abuse of prescription drugs has its own set of consequences and a lethal toxicity. Prescription drug abuse is prevalent in many areas around the world including southern Africa where there is limited research. The Foundation of a Drug Free World reported international statistics that indicate that opioids, depressants and antidepressants lead to more overdose deaths (45%) compared to cocaine, heroin, methamphetamine and amphetamines (39%) combined.

According to the South African Community Epidemiology Network on Drug Use (SACENDU), benzodiazepines are some of the most commonly misused hypnotic sedatives that act as depressants of the central nervous system (CNS). Negative side effects such as disinhibition, psychomotor retardation, emotional blunting and cognitive dysfunction have been found to be in association with the illicit abuse of this class of drugs (SACENDU, 2014). Moreover, concurrent misuse with other CNS depressants, such as heroin and alcohol, highly increases the likelihood of motor vehicle accidents and overdose or death of users.

The long-term result of benzodiazepine abuse is development of dependence to the drugs on both a psychological and physical level (SACENDU, 2014). Due to the sedative effects that this drug class induces in individuals, it is also frequently used for drug facilitated sexual assault (Pal &Teotia, 2010). Flunitrazepam for instance is a strong sedative that is one of the most well-known date rape drugs. It is typically referred to as ‘roofies’ or ‘the forget pill’ as it also causes short-term memory loss (Pal &Teotia, 2010). Taking these uses and effects into consideration, the drug class of benzodiazepines are thus important in clinical and forensic toxicology.

Another group of prescription drugs that also acts on the central nervous system of individuals is antidepressants. This class of drugs are typically used for the treatment of depression and other psychiatric disorders affecting the everyday lives of many people. As stated by Titier*et al.* (2007), the use of antidepressants is a main cause of voluntary life-threatening intoxication and suicide amongst patients. It has also been reported that fatal antidepressant overdose can occur as a mishap and is not necessarily always intentional

(Koski, 2005). Furthermore there is wide range of variability in the metabolism of antidepressants between individuals and therefore drug dosage should be optimized for each individual patient (Ostad*et al.*, 2012). This personalised drug dosage could aid in the prevention of the occurrence of unfavourable side-effects associated with antidepressants use such as cardiac disturbances, respiratory depression, metabolic acidosis, convulsions and comas (Jickells&Negrusz, 2008). Due to these scenarios, antidepressant consumption is also significant in clinical and forensic toxicology.

Benzodiazepines and antidepressants are commonly encountered prescription drugs and their toxicological analysis is essential to aid in modern death and forensic case investigation. Several methods have been published for the determination of these types of drugs in different biological matrices (Remane*et al.*, 2010 Remane*et al.,* 2011, Johnson-Davis *et al.,* 2012; Sauve*et al.*, 2012 Verplaetse *et al.*, 2012Bjørk*et al.*, 2013,Hegstad*et al.*, 2014, Montenarh*et al.,* 2014a, b, c). These methods describe liquid chromatography tandem mass spectrometry (LC-MS/MS) techniques as a method of screening and quantification of the analytes. LC-MS/MS is appropriate for the analysis of a large number of compounds including thermo-labile and polar compounds without the need for initial derivitization steps which is advantageous over the considered gold standard gas chromatography mass spectrometry (Peters, 2011). The high sensitivity associated with LC-MS/MS analysis makes it a suitable approach for the simultaneous determination of an extensive range of analytes.

LC-MS/MS methods targeting multiple analytes, rather than a single or a few analytes, are highly applicable tools in clinical and forensic toxicology to broaden the range of drugs that can be screened for simultaneously in a short period of time using a low sample volume. Multi-analyte approaches reduce time and costs involve for analysis and it is thus beneficial for laboratories to develop such LC-MS/MS methods. Validation of a developed LC-MS/MS quantification procedure is essential to establish whether the procedure can indeed determine the analytes of interest in biological matrices in a fixed and reliable manner. In order for the application of the bio-analytical method in routine practices, it should be well characterised, fully validated and documented to a satisfactory standard (EMEA/CHMP/EWP/192217/2009).

In the current study, a multi-analyte LC-MS/MS method is described for the detection and quantification of 11 commonly encountered analytes from both benzodiazepine and antidepressant drug classes in blood, serum and urine from therapeutic to overdose concentrations. The quantification procedure in blood could only be partially validated due to time constraints for the project. Authentic samples of each of the three biological matrices were analysed with the developed method to investigate its toxicological suitability.

2. Materials and Methods

2.1 Chemicals and Reagents

A total of 11 drugs were used in this study, namelyeight benzodiazepines: alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, tenazepam; 2 tricyclic antidepressants: amitriptyline and nortriptyline; and the selective serotonin re-uptake inhibitor (SSR) antidepressant, citalopram. A benzodiazepine Multi-Component Mixture-8 stock standard solution was prepared in acetonitrile at a concentration of 250 µg/mL (Cerilliant Corporation, Round Rock, Texas, USA). Stock standard solutions for each of the three antidepressant analytes were also obtained from Cerilliant Corporation (Round Rock, Texas, USA). This included amitriptyline hydrochloride stock standard at a concentration of 1.000 mg/mL (as free base) prepared in methanol; nortriptyline hydrochloride stock standard at a concentration of 1.000 mg/mL (as free base) prepared in methanol and citalopram, primary standard at a concentration of 100 µg/mL prepared in methanol. Working solutions of 100 ng/mL, 1 µg/mL, 10 µg/mL and100 µg/mL of the 11 compounds conjoined was prepared in distilled water. Solutions were stored at a temperature of – 80 °*C when it was not in use.* The Forensic Drug Screen Internal Standard Solution #1, containing deuterated doxepin-d_3 and diazepam-d_5, was used as internal standard and was obtained from Restek (Bellefonte, USA) at a concentration of 10 µg/mL in methanol. An internal standard working solution of 80 ng/mL was prepared in acetonitrile as the precipitating reagent and it was stored at – 4 °*C*when it was not utilized. The purity of all reference standards was more than 99%.

2.2 Specimens

Drug-free blood and serum was obtained from the Western Province Blood Transfusion Services (WPBTS) and urine was obtained from a drug-free volunteer in the Division of Pharmacology at the University of Cape Town for validation purposes. Post-mortem and clinical samples submitted to the authors' laboratory for routine toxicology analysis that tested positive for any of the target analytes in this study were used as authentic samples to test the applicability of the developed LC-MS/MS quantification method.

2.3 Apparatus

The liquid chromatographic system consisted of a Shimadzu Prominence HPLC (Shimadzu Corporation, Kyoto, Japan) which comprised of a degasser, a binary pump and an autosampler. The needle in the autosampler was rinsed before and after injection for 5 seconds. The analytical detection system consisted of an Applied Biosystems API 3200^{TM} Triple Quad MS/MS System (ABSCIEX, Toronto, Canada)operated in multiple reaction monitoring (MRM) mode and positive electrospray ionization (ESI).

2.4 HPLC Conditions

Gradient elution was performed on a Kinetex C18 (50 mm x 3 mm, 2,6µm particle size) column from Phenomenex(California, USA). The mobile phases consist of 2mM ammonium acetate (Merck, Darmstadt, Germany) with 0.2 % formic acid in ultrapurified water (aqueous eluent A) and 2mM ammonium acetate with 0.2 % formic acid (Fluka Analytical, Sigma Aldrich, USA) in gradient grade acetonitrile (Merck, Darmstadt, Germany)as the organic eluent B. Fresh mobile phases were prepared on a regular basis and filtered through a Synergy Water Purification System (Millipore, France). Gradient elution was programmed to run from 1% organic eluent B to 95 % organic eluent B in 6 minutes. The organic phase concentration was then decreased back to 1% from 6.1 minutes to 9.4 minutes with a total run time of 9.5 minutes. The flow rate was 0.3 mL/min and the column oven was maintained at 40 °C.

2.5 MS/MS Conditions

For detection and quantification, the following electrospray ionization (ESI) inlet conditions were applied: gas 1 at 35 psi; gas 2 at 70 psi; ion-spray voltage, 5000 V; source temperature, 450 °C; curtain gas at 25 psi. The dwell time for each analyte was 40 milliseconds. All data and chromatograms were analysed with Analyst 1.6.2 software (ABSCIEX, Toronto, Canada). Direct infusion of astandard reference solution at 100 ng/ml (diluted with 50 % eluent A and 50 % eluent B) was performed andprecursor ions were selected and fragmented from resulting spectra. Two MRM transitions (two product ions) per analyte (precursor ion) were monitored in the compound optimization mode and the most abundant MRM transition was considered as the quantifier ion and the second most abundant transition as the qualifier. Flow injection analysis (FIA) was used to optimise the mass spectrometry parameters.The mass-to-charge ratios of theprecursor ion and product ions for each analyte are indicated in Table 1 as well as mass spectrometric conditions set for each ion which was determined by FIA such asthe declustering potentials, collision energies and collision cell exit potentials for each analyte.

2.6 Calibration Standard and Quality Control Preparation

All pipettes were verified before the start of experiments. Calibration standards were prepared by spiking samples of blank blood, serum and urine with the highestconcentration of standard (2000 ng/mL) and then serial diluting the samples with blank blood, serum or plasma to attain the desired concentrations of eight calibration standards: 15.6, 31.2, 62.5, 125, 250, 500, 1000 and 2000 ng/mL. The concentration range was selected based on published literature for the purpose of investigating overdoses or toxic levels of the drugs. The lower limit of quantitation (LLOQ) was established to be 15.6 ng/ml and extra aliquots were stored to utilize as quality controls. Multiple 100µL aliquots of each calibration standard were stored in individual 1.5mL polypropylene tubes at – 80 °*C* when not in use. During validation, the calibrators were thawed and prepared as described in section 2.7 below before LC-MS/MS analysis.Replicates (n=2) at each calibration level were analysed three times over threedays for blood and two days for serum and urine. Calibration curves were obtained by plotting the peakarea of analytes to internal standard versus the analyte concentration to internal standardusing a weighted (1/x) quadratic regression model.

Table 1: Precursor ion and product ions mass-to-charge ratios for each analyte and declustering potential, collision energy, and collision cell exit potential set for each ion during mass spectrometric detection

Analyte	Q1 (m/z)	Q3 (m/z)	DP (V)	CE (V)	CXP (V)
Nortriptyline	264.2	233.2	51	19	4
		91.1	51	33	2
Amitriptyline	278.3	91.2	51	50	2
		105.1	51	50	2
Citalopram	325.2	109.1	51	50	2
		262.2	51	50	2
Alprazolam	309.1	281.2	81	41	4
		205.1	81	55	4
Clonazepam	316.0	270.0	71	37	4
		302.2	71	37	4
Diazepam	285.2	154.1	76	37	4
		193.2	76	43	4
Flunitrazepam	314.3	268.2	76	31	4
		300.2	76	31	4
Lorazepam	323.1	277.1	71	29	4
		305.1	71	19	4
Nitrazepam	282.1	180.2	71	53	4
		236.1	71	37	4
Oxazepam	287.1	241.1	66	31	4
		269.1	66	19	4
Temazepam	301.1	255.2	51	31	4
		283.1	51	17	4
Diazepam-d_5	290.1	198.2	71	47	4
Doxepin-d_3	283.2	107.1	61	33	2

CE - Collision Energy; CXP - Collision Cell Exit Potential; DP - Declustering Potential; m/z - mass to charge ratio; Q1 - Precursor Ion; Q3 - Product Ion; V - volts

Low (L QC), medium (M QC) and high quality controls (H QC) were prepared in all three matrices by spiking blank blood, serum or urine with the highest concentration of the calibration standard in such a manner to obtain 45ng/mL (L QC), 800ng/mL (M QC) and 1600 ng/mL(H QC) respectively. Extra M QCs were stored for the purpose of performing a system suitability test during validation. Multiple 100µL aliquots of each QC (including the LLOQ) were stored in individual 1.5mL polypropylene tubes at – 80 °*C* when not in use. During validation, the QCs were thawed and prepared as described in 2.7 below before LC-MS/MS

analysis. Quality controls were run with each calibration curve in order to assess accuracy and precision as well as n number of other validation parameters for blood which will be explained further on in section 3.

2.7 Sample Preparation

2.7.1 Blood and Serum

Blood and serum samples were prepared using similar protein precipitation method for both matrices. A volume of 25 µL of water was added to100 µL of a blood or serum sample in a 1.5 mL Eppendorf tube (Melbourne, Australia). After vortexing the mixture swiftly for about 5 counts, 500 µL of precipitating reagent, namely cold acetonitrile (Merck, Darmstadt, Germany)(with 80 ng/ml internal standard), was added and the samples were vortexed for another 5 counts. Samples were thenleft to be sonicated with a water bath UMC5 sonicator (Instrulab, United States of America) for 10 minutes before centrifuging at 13 000 rpm for five minutes using a Biofuge 13 benchtop centrifuge (Canada). A volume of 500 µL of thesupernatant of each sample was removed and dispersed into 5mL Kimble tube which was then placed into a Stuart Sample Concentrator Block Heater (Staffordshire, UK) for evaporation at 40 °*C*under a constant flow of nitrogen gas (20 kPa) for 20 minutes. The residue was reconstituted with 200 µL LC-MS grade water and 150 µL was transferred to 96-well plates which were then sealed. Ten microliters were injected into the HPLC-MS/MS system for analysis.

2.7.2 Urine

In a 1.5 mL Eppendorf tube (Melbourne, Australia) 100 µL of a urine sample was mixed with 100 µL of the precipitating reagent, cold acetonitrile (with 80 ng/ml internal standard). The samples were vortexed for about 5 counts after which it was centrifuged for 10 minutes before centrifuging at 13 000 rpm for five minutes (with a Biofuge 13 benchtop centrifuge). A volume of 200 µL of the supernatant was pipetted into a new 1.5 mL Eppendorf tube (Melbourne, Australia) containing 400 µL LC-MS grade water. Tubes were vortexed again for 5 counts before 150 µL was transferred into autosampler glass vials. Tenmicroliters were injected into the HPLC-MS/MS system for analysis.

3. Methodology for Validation Experiments

Validation guidelines for bio-analytical methods provided by the European Medicines Agency (EMEA) in combination with those provided by the U.S. Food and Drug Administration (FDA) were used as international recommendations to compile a Bioanalytical Method Validation Standard Operating Procedure(SOP) document for the use in the Division of Pharmacology at the University of Cape Town revised by Castel 2014). It includes details on how to perform different validation experiments along with the acceptance criteria for resulting data.

3.1 Accuracy and Precision

Accuracy can be defined as the closeness of the mean test results obtained by the method to the actual concentration value of the analytes. Precision is an indication of the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of the same sample and thus gives an indication of the reproducibility of results. Intra-batch precision and accuracy is an assessment of precision and accuracy during a single analytical run whilst Inter-batch precision and accuracy is an assessment of precision and accuracy over time. (Castel, 2014)

3.1.1 Blood

Within-batch accuracy and precision were assessed by assaying all eight concentration levels of calibration standards in duplicate to produce one calibration curve along with 6 duplicates of each quality control level (LLOQ, L QC, M QC and H QC). Accuracy and precision as within-batch validation parameters were assessed by calculating the quadratic regression (weighted by 1/x concentration) equation and constructing the calibration curve based on peak area ratios of analyte to internal standard (Castel, 2014).

Between-batch accuracy and precision were assessed by assaying an additional two separate consecutive batches of curves, each consisting of a double set of calibration standards with six replicates of each of the quality control standards. The between-batch accuracy and precision of the assay procedure were also assessed by constructing a

calibration curve based on analyte/ISTD (internal standard) peak area ratios and calculating the regression equations (Castel, 2014).

The overall accuracy and precision of the assay procedure is assessed by calculating the accuracy and precision statistics over the within-and between batch validation batches (n = 3). Accuracy is expressed as the concentration of the analyte as a percentage of the nominal concentration (%Accuracy), while precision is expressed as the coefficient of variation (%CV). (Castel, 2014)

Concentrations determined with the developed method for calibration standards (STDs) should have a %Accuracy within 15% of the nominal (or expected) concentration (85 – 115 %), except for the LLOQ, which should be within 20% (80 – 120 %) of the nominal value. The same criteria are applied to QCs and 67% of the total QCs in each validation run should pass the above criteria. At least 75% of the total STDs in each validation run should meet the above criteria (i.e. 6 out of the 8 points on the calibration curve) and also at least 50% of the STDs tested at each concentration level must meet acceptance criteria. (Castel, 2014)

3.1.2 Serum and Urine

In contrast to the calibration of blood, two replicates of each quality control level were analysed instead of six with each duplicate calibration curve for these matrices. The second and third set of calibration curves for these matrices could therefore both be run on day 2 of validation for both matrices respectively.

3.2 Application to Authentic Samples

The extraction and LC-MS/MS method developed for each type of biological matrix by this project were verified by analysing authentic samples. These samples were submitted to the authors' laboratory for routine testing, either via immunoassay techniques or general unknown screening via LC-MS/MS. Samples in which any of the 11 target analytes were detected were consequently analysed with the developed quantification LC-MS/MS method. All authentic samples were prepared as described in section 2.7. Table 2 below summarises the five different authentic samples that were tested, including the initial screening result found for each sample (underlined). It should be noted that both 'Blood

Unknown 1' and 'Urine Unknown 1' were collected during an autopsy of an individual who possibly died from a drug overdose.

3.3 Carry-over Effect

A double blank sample (without analyte and IS) was positioned in the injection sequence immediately after the highest calibration standard in order to assess possible carry-over effects. A blank sample (without analyte) was also included to determine the possible contamination of the analyte by the internal standard without an additional carry-over effect. (Castel, 2014)

Table 2: Authentic samples used to verify the developed quantification LC-MS/MS method and results found after initial screening with immunoassay or LC-MS/MS general unknown screening

Matrix	Type of sample	Name	Initial Screening Result
Blood	Forensic Post-mortem sample	Blood Unknown 1	GUS via LC-MS/MS: Amitriptyline detected
Serum	Clinical sample	Serum Unknown 1	Immunoassay: Positive for TCAs
Urine	Forensic Post-mortem sample	Urine Unknown 1	GUS via LC-MS/MS: Amitriptyline detected
	Clinical sample	Urine Unknown 2	Immunoassay: Positive for BDPs
	Clinical sample	Urine Unknown 3	GUS via LC-MS/MS: Citalopram detected

BDP – benzodiazepines; GUS – general unknown screening; LC-MS/MS – liquid chromatography tandem mass spectrometry; TCAs – tricyclic antidepressants

Further descriptions of validation experiments that follow (3.3 to 3.8) were only performed on blood samples.

3.4 Sensitivity

Six different blank sources of blood (A - F) were extracted without including internal standard in the precipitating agent and were then spiked at the LLOQ concentration level of 15.6 ng/mL, taking into account any calculations for dilutions in the analytical method. The six different blank sources of blood included three clinical blank blood sources and three post-mortem blank blood sources. Acceptance criteria for sensitivity include that the mean analyte signal/noise response at LLOQ of the six samples should at least be five times the response compared to the blank response at the retention time of interest. (Castel, 2014)

3.5 Recovery

The extraction recovery was determined by comparing the analytical response of blank blood spiked with analytes and extracted (referred to as test samples) with the response of the blank matrix first extracted and then spiked with analytes (theoretical samples) taking into account calculations for dilutions in the analytical method. Three QCs at each concentration level (low, medium and high) were extracted as per the analytical method to represent test samples and theoretical samples were spiked at each concentration level in triplicate using extracted blank matrix. International acceptance criteria for recovery of a quantitative drug assay include a consistent mean recovery and a percentage coefficient of variation (% CV) should ideally be lower than 15% for a precise extraction recovery. Furthermore, the recovery reproducibility between concentration levels should also be less than 15% (Castel, 2014).

3.6 Matrix Effects

In order to assess the effects of components present in the blood matrix other than the analytes, six different blank sources of blood (A - F) were extracted without IS. The six different blank sources of blood are from the same sources as described for the sensitivity experiment in section 3.4. Each individual matrix was then spiked with low, medium and high QC concentration levels (taking into account any calculations for dilutions in the analytical method) and at one concentration of the ISTD before analysis. Peak area ratios of the analyte/IS for each level in each matrix source are used to generate regressions for each

individual matrix. The slope variability (% CV) for the six different matrix sources should not be more than 5%.

3.7 Stability

Stability of the analytes in blood was tested by leaving low, medium and high QCs in duplicate on the laboratory bench at a room temperature of 25 °C and at 4 °C in the refrigerator for one week. The average peak areas for each of the duplicate QC levels were compared to the average peak areas obtained from freshly prepared QCs at each concentration level in duplicate. The peak areas obtained for the different analytes in in freshly prepared samples were assumed to resemble the peak areas resulting from stable analytes running through the system.

4. Results and Discussion

4.1 HPCL-MS/MS Method Development

Chromatographic separation as seen in Figure 1 below was achieved by optimizing various chromatographic conditions including the analytical column used, the mobile phases and their composition gradient as well as the flow rate setting. Initially an aqueous mobile phase of 10mM ammonium formate in water and an organic phase of 50% acetonitrile:methanol was tested with a steep gradient of 2%organic phase increasing to 95% in the first three minutes of the run and a total run time of five minutes. A gradient of 5% and 10% organic phase increasing to 95% was also respectively tested but ultimately a gradient of 1% organic phase increasing to 95% was selected. Due to the amount of target compounds in the method, the run time was ultimately increased to 9.50 minutes which allows for adequate equilibration of the system as well. The flow rate was set at 0.5 ml/min with an injection volume of 5 µL. After optimization of the sample preparation method, it was decided to set the injection volume at 10 µL for adequate peak intensities.

An Allure PFP Propyl column (50 mm x 2.1 mm, 5 µm) (Restek, Pennsylvania, USA) was used and the oven temperature was set at 60 °C. After flow injection analysis, a lower oven temperature of 40 °C was found to be acceptable. This is advantageous as it takes less time for the instrument to preheat the oven. More reproducible chromatography was achieved

by the utilization of a Kinetex C18 analytical column (50 mm x 3 mm, 2.6 µm) (Phenomenex, California, USA) and a flow rate of 0.3 ml/min was found to create less pressure for the smaller column. This is the same column used for routine drug screening by LC-MS/MS by the authors' laboratory and it was thought to be favourable to use the same column due to ease of switching over to the quantification method should any of the target analytes be detected during screening.

For the same reason it was decided to also utilize the same mobile phases as that used for routine drug screening. Various different combinations of mobile phases with the two columns were however tried and tested during method development. Ammonium formate as an aqueous mobile phase did not give optimal chromatography and thus chromatography with ammonium acetate at a concentration of both 10 mM and 2 mM in water with 0.2% formic acid as an acidic additive for positive ionization was investigated. For the organic phase, acetonitrile and methanol with 0.1 % formic acid was also tested. Ultimately the drug screen combination of 2mM ammonium acetate with 0.2 % formic acid in water and 2mM ammonium acetate with 0.2 % formic acid in acetonitrile showed acceptable separation.

Figure 1presents a chromatogram to show the separation of an extractedblood standard containing all 11 compounds at a concentration of 800 ng/mL by the developed LC-MS/MS method. Each compound is presented by their quantifier ions which are shown by different coloured peaks. The compounds are detected between 4.67 minutes and 5.72 minutes after injection of the standard sample and the retention time (Rt) for each compound is indicated.

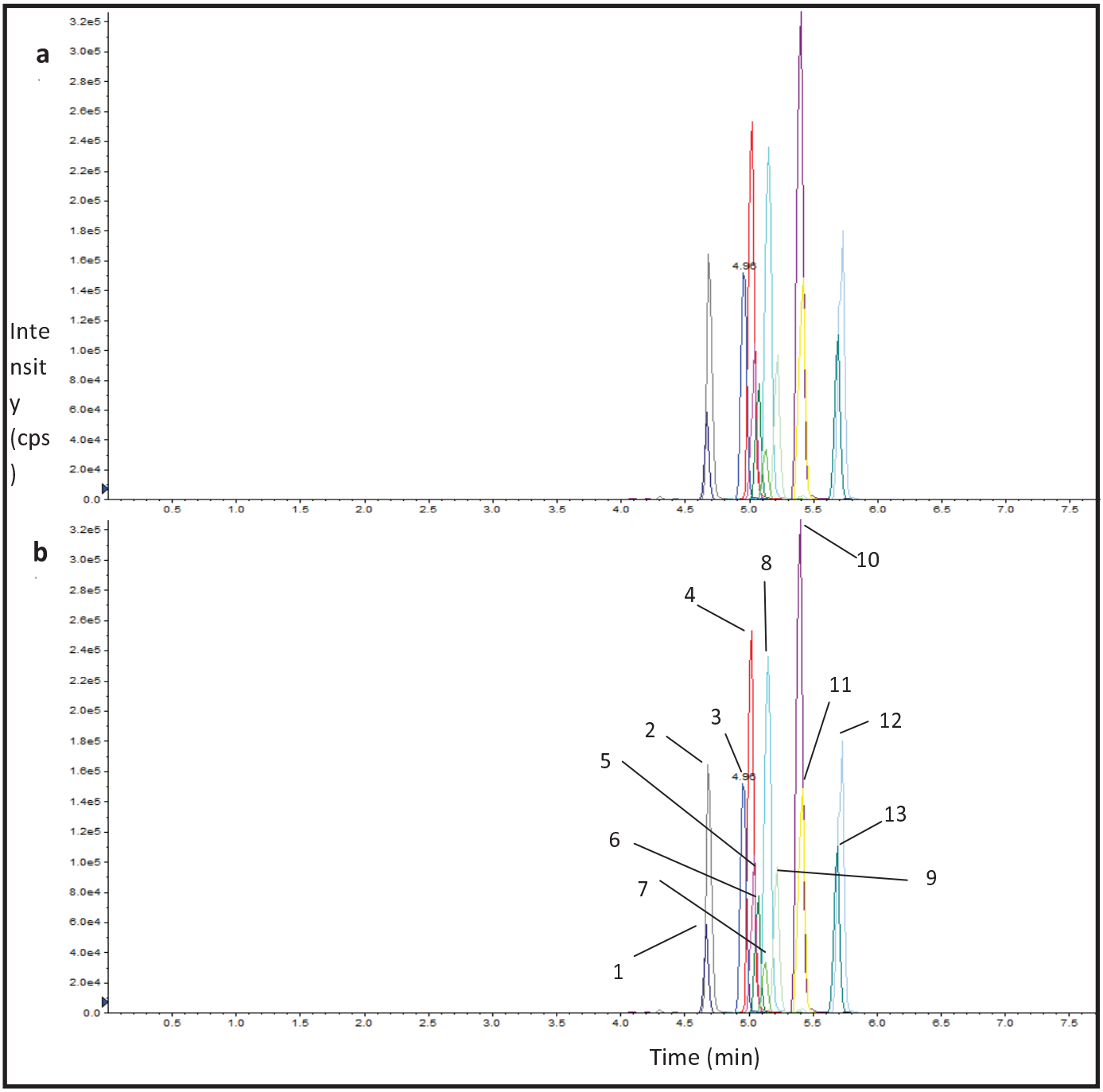


Figure 1: a: Chromatogram of an extracted blood standard at 800 ng/mL.b: The same chromatogram with quantifier ions numbered for each analyte. 1 - Citalopram (dark blue, Rt: 4.67 min); 2 - Doxepin-d_3 (grey, Rt: 4.69 min); 3 - Nortriptyline (royal blue, Rt: 4.96 min); 4 - Amitriptyline (red, Rt: 5.02 min); 5 - Oxazepam (pink, Rt: 5.04 min); 6 - Nitrazepam (dark green, Rt: 5.07 min); 7 - Lorazepam (light green, Rt: 5.13 min); 8 - Alprazolam (turquoise, Rt: 5.15 min); 9 - Clonazepam (light grey, Rt: 5.22 min); 10 - Temazepam (purple, Rt: 5.40 min); 11 - Flunitrazepam (yellow, Rt: 5.41 min); 12 - Diazepam-d_5 (teal, Rt: 5.68 min); 13 - Diazepam (light blue, Rt: 5.72 min)

4.2 Accuracy and Precision

The results for the accuracy and precision validation for blood, serum and urine are displayed in Appendix A, B, and C respectively. A representative calibration curve for each analyte (indicating the r^2 fit parameter) is presented followed by the calibration standard and quality control accuracy and precision results for each analyte per calibration curve (validation 1 to 3). The combined calibration standards and quality control results for each analyte over the three validation runs are also represented.

Results from the validation assays indicate a valid calibration range of 15.6 – 2000 ng/mL for all analytes.The LLOQ was set at the concentration of the lowest validated standard for each analyte, namely 15.6 ng/mL.Calibration curves for all matrices were found to be linear over the range tested with r^2 values greater than 0.95. In Appendix A, B and C, %Accuracy values that did not meet the acceptance criteria were highlighted in grey and Tables A 45, B 45 and C 45 at the end of each appendix provides a summary of these values. These tables also indicate the amount of percentage with which the values were too high or too low to meet either the lowest or highest accepted accuracy values. Looking at the overall accuracy and precision of the assay procedure in each matrix over the three validation batches, it can be seen that in most cases the overall %Accuracies for STDs and QCs did meet the acceptance criteria (Castel, 2014). In cases where it was not met, the %Accuracy is not out by more than 8%. Results for oxazepam in blood however did not meet acceptance criteria to a high degree and must be rejected (Castel, 2014). The results show that the method provides sufficient accuracy and precision over the entire range based on analyte/internal standard peak area ratios with a quadratic calibration curve (weighted by 1/x concentration).

4.3 Application to Authentic Samples

4.3.1 Blood Unknown 1 and Urine Unknown 1 both from Autopsy Case

Clinical and post-mortem forensic samples sent to the laboratory can approximately be quantified if any of the 11 analytes targeted by the developed quantification LC-MS/MS method is detected. One post-mortem blood sample and one post-mortem urine sample collected from the same individual was quantified after the presence of amitriptyline was detected. The quantification results are displayed in Table 3 below.

Table 3:Quantification results of 'Blood Unknown 1' and 'Urine Unknown 1' post-mortem samples that were both collected from a deceased individual during autopsy and tested positive for the presence of amitriptyline after routine drug screen by HPLC-MS/MS

Authentic Sample	Dilution	Quantification Result (ng/mL)	
		Amitriptyline	Nortriptyline
Blood Unknown 1	1:1	No intercept	134
	1:4	1970	91.3
Urine Unknown 1	1:1	1410	118.5
	1:4	606	48.95

ng/mL – nanogram per millilitre

Both amitriptyline and nortriptyline were detected in both the post-mortem blood and urine samples. This correlates to the detection of amitriptyline during the routine drug screen by HPLC-MS/MS. The presence of nortriptyline in a lower concentration could be expected as nortriptyline is a metabolite of amitriptyline (Johnson-Davis *et al.,* 2012). It can be seen that both the blood and urine samples contained a high concentration of amitriptyline. In the 1:4 diluted blood sample, the amitriptyline concentration was found to be 1970 ng/mL and 1410 ng/mL in the 1:1 diluted urine sample. These determined concentration values are close to the highest calibration concentration of 2000 ng/mL. Samples thus had to be diluted with blank matrix to fall within the calibration range of the developed LC-MS/MS method. The 1:1 diluted blood sample had no intercept as the concentration of amitriptyline in this sample was higher than the highest point on the calibration curve. Quantification of amitriptyline in blood could only be carried out in the 1:4 diluted sample as this dilution caused the concentration to fall within the calibration range. The method found the blood amitriptyline concentration to be 1970 ng/mL in the 1:4 diluted sample which correlates to 7880 ng/mL in undiluted blood.

By making reference to the comprehensive list published by Schulz *et al.* (2012) containing reported therapeutic and toxic plasma concentration ranges (included in Chapter 2), it can be seen that reported comatose to fatal concentration levels of amitriptyline include 1500 to 2000 ng/mL in serum. Blood and serum levels can roughly be compared taking into

account factors to consider when comparing post-mortem drug levels to published *in vivo* drug levels (Linette, 2012). It is however projected that the level of amitriptyline in the deceased individual was indeed in a toxic range based on the high concentration determined for amitriptyline with the developed method. It can possibly be speculated that it was related to the death of the individual. This should especially be considered as amitriptyline antidepressants were found at the crime scene next to the deceased. The concentration levels of amitriptyline and nortriptyline found in urine was lower than that found in blood - 606 ng/mL in the 1:4 diluted urine sample compared to 1970 ng/mL in the 1:4 diluted blood sample.

4.3.2 Other Authentic Samples Quantification Results

Table 4 that follows show the quantification results obtained for the other three authentic samples that were analysed. A similar trend as seen in the above mentioned autopsy case is observed for the quantification results of 'Serum Unknown 1' with a higher concentration of amitriptyline (891 ng/mL) versus nortriptyline (212 ng/mL). The LC-MS/MS method was shown to produce more specific results regarding the presence of both amitriptyline and nortriptyline as opposed to the confirmation of the presence of tricyclic antidepressants as a drug class using the immunoassay test. Immunoassay testing was unable to distinguish or estimate the analyte concentrations as was achieved by the LC-MS/MS method.

Similarly, the sample 'Urine Unknown 2' also showed that the LC-MS/MS method could indicate the presence of the antidepressant citalopram, whereas the immunoassay only resulted in a positive test for benzodiazepines as a drug class. The LC-MS/MS method did result in a chromatogram that indicated the presence of certain benzodiazepines in this urine sample including lorazepam, oxazepam and temazepam. The presence of these benzodiazepines, though confirmed, could however not be quantified as their retention times did not correlate to that found via the developed LC-MS/MS method. Citalopram could be quantified and it was found to be present at a concentration of 1600 ng/mL.Schulz *et al*. (2012) reported a concentration range of 200 to 1500 ng/mL of citalopram in serum to be a normal therapeutic level. Thus roughly comparing these values, it can possibly be said that the individual is using citalopram as a prescription drug and it is not necessarily a case of abuse and negative side-effects are not likely to be an occurrence.In the case of 'Urine

Unknown 3', it can be seen that a very high concentration of citalopram was determined, i.e. 5900 ng/mL. Comparing this to serum concentrations of citalopram presented by Schulz *et al.* (2012), it would indicate comatose or fatal levels. This is not likely as this authentic sample was collected from a living individual. Quantification of urine samples is not optimal to deduct pharmacological effects. The presence of citalopram was still confirmed after immunoassay detection with a peak of high intensity.

Table 4: Quantification results obtained for 'Serum Unknown 1', 'Urine Unknown 2' and 'Urine Unknown 3'

Authentic Sample	**Initial Screening Result**	**Quantification Result (ng/mL)**	
Serum Unknown 1	Immunoassay: Positive for TCAs	Amitriptyline	Nortriptyline
		891	212
Urine Unknown 2	Immunoassay: Positive for BDPs	No DBPs detected, Citalopram however detected	Citalopram
			1600
Urine Unknown 3	GUS via LC-MS/MS: Citalopramdetected	Citalopram	
		5900	

BDPs – benzodiazepines; GUS – general unknown screening; LC-MS/MS – liquid chromatography tandem mass spectrometry; ng/mL – nanogram per millilitre; TCAs – tricyclic antidepressants

Due to only three analytes, namely amitriptyline, nortriptyline and citalopram quantified, validation results that follow are only presented for these compounds and not for all 11 original compounds.

4.4 Carry-over Effect

No noteworthy carryover was observed in chromatograms. Representative chromatograms of a double blank sample in each of the matrices assessed is presented in Figure 2 followed by representative chromatograms of a blank sample in each matrix presented in Figure 3.

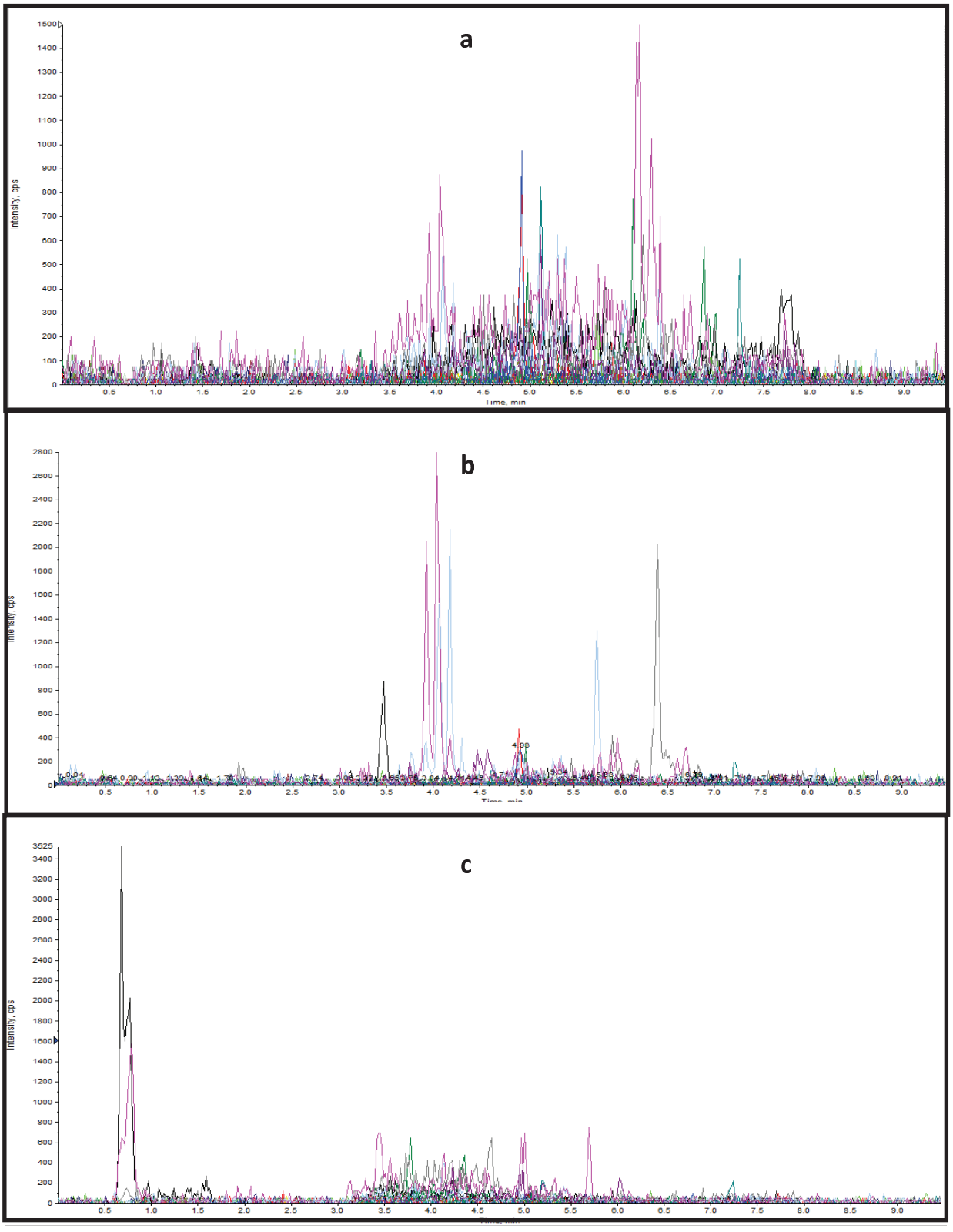


Figure 2: Chromatogram of a double blank sample in blood (a), serum (b) and urine (c).

A double blank sample does not contain any analytes or internal standard and it thus expected to see no sharp peaks that indicate the presence of any analytes in the resulting

chromatogram. A double blank sample was injected immediately after the injection of the highest standard at 2000 ng/mL in order to assess whether analytes are possibly carried over into the following injection and detected as a false positive as a result. Figure 2 shows only background peaks at a very low intensity (max 975 cps) and no analytes are detected.

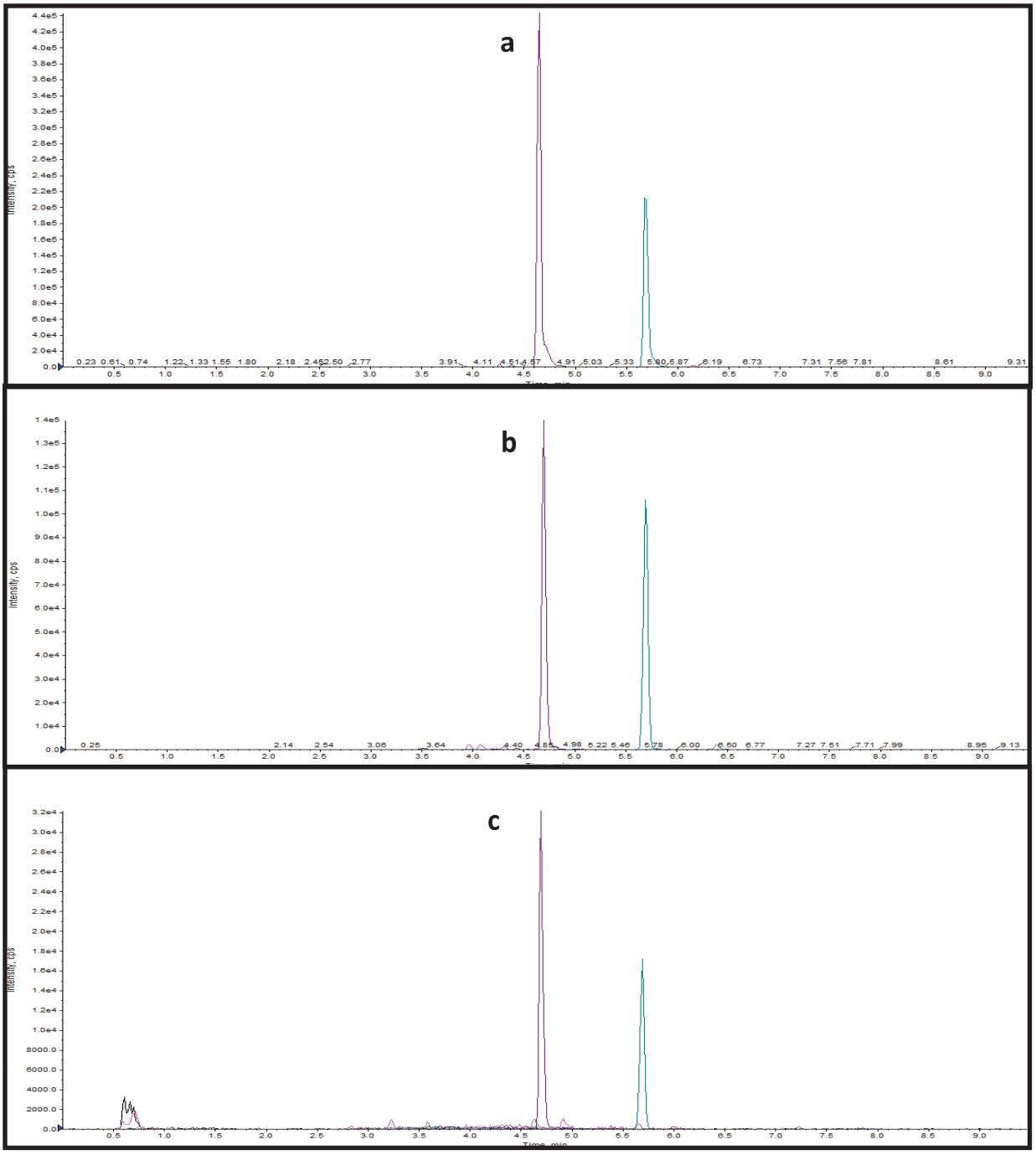
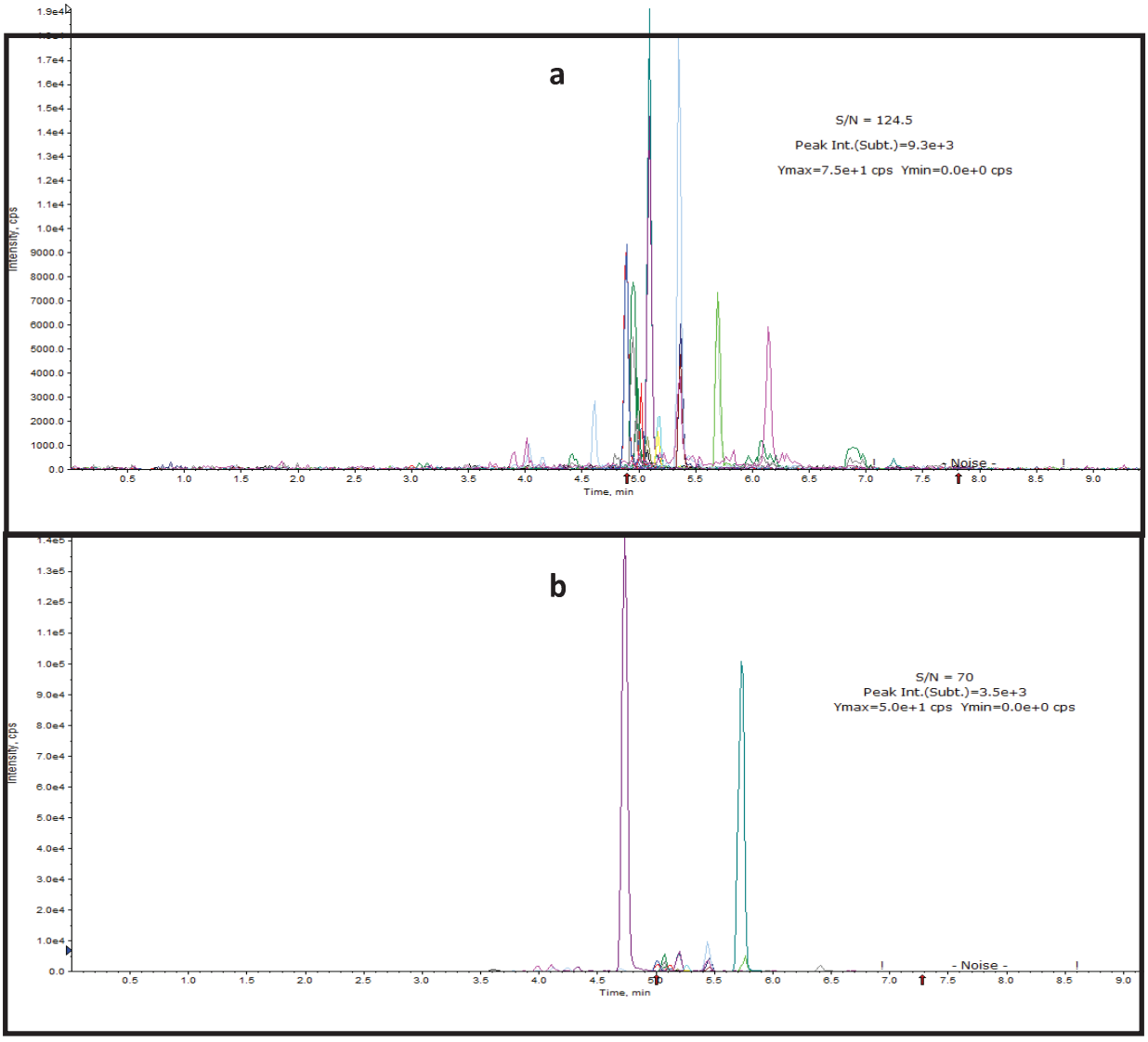


Figure 3: Chromatogram of a blank sample in blood (a), serum (b) and urine (c).

4.5 Sensitivity

The LLOQ of this method was selected to be 15.6 ng/mL as this was the eighth point for the calibration curve when serial diluting down from 2000 ng/mL. This is also in accordance to the Bioanalytical Method Validation SOP that the calibration curve should at least have six non-zero different concentration levels covering the entire calibration range. Eight calibration levels are therefore sufficient should one or two of the calibration levels of the curve not meet the acceptance criteria. There was also not a need to have lower concentration levels than this LLOQ level as the method would principally be utilised to investigate overdose situations. Figure 4 below is a representative chromatogram of the signal/noise ratio for a LLOQ sample in each matrix assessed.



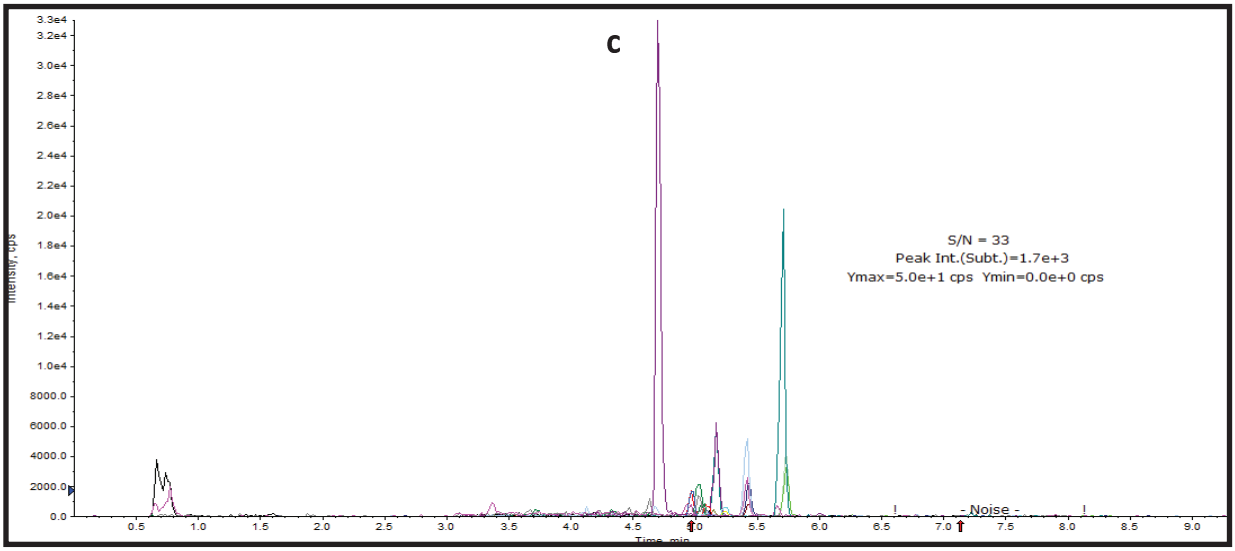


Figure 4: Representative chromatogram of the signal/noise(S/N) ratio for a LLOQ sample in (a) blood, (b) serum and (c) urine.

It can be seen that the S/N ratio in blood at LLOQ (Figure 4a) is 124.5 is much more than the acceptance criteria of five times the response compared to the blank response around the retention time of interest. The raw LLOQ sample chromatograms showed acceptable intensities for the analytes with an average S/N ratio of 163.9 for the analytes (n = 6). The instrument can therefore be suggested as sensitive enough to detect the level of the LLOQ concentration.

4.6 Recovery

The ratios of analyte/ISTD peak area found after extraction of test samples are compared to the theoretical sample peak area ratios and are expressed as a percentage recovery. The tables below show the calculated extraction recovery efficiencies for three antidepressants. The recovery need not be 100%, but should be consistent with recovery reproducibility between concentration levels not greater than 15% and %CV values also lower than 15%.

Table 5: Recovery for Amitriptyline

	High Concentration (1600 ng/mL)		Medium Concentration (800 ng/mL)		Low Concentration (45 ng/mL)	
	Test Sample: Peak Area	Theoretical Sample: Peak Area	Test Sample: Peak Area	Theoretical Sample: Peak Area	Test Sample: Peak Area	Theoretical Sample: Peak Area
Sample 1	2720000	2500000	1550000	1260000	95100	92100
Sample 2	2680000	2330000	1370000	1560000	98300	65400
Sample 3	2620000	2280000	1540000	1460000	101000	90000
Average	2673333	2370000	1486667	1426667	98133.33	82500
STDEV	50332.23	115325.6	101159.9	152752.5	2953.529	14846.21
% CV	1.882752	4.86606	6.80448	10.70695	3.00971	17.99541
% Recovery		**112.8**		**104.2**		**119**
				Avg. % Recovery		**112**
				Avg. % CV		**7.6**

The mean recovery of amitriptyline from blood over the calibration range is 112 % with a % CV of 7.6 and meets the criteria for adequate recovery.

Table 6: Recovery for Amitriptyline

	High Concentration (1600 ng/mL)		Medium Concentration (800 ng/mL)		Low Concentration (45 ng/mL)	
	Test Sample: Peak Area	Theoretical Sample: Peak Area	Test Sample: Peak Area	Theoretical Sample: Peak Area	Test Sample: Peak Area	Theoretical Sample: Peak Area
Sample 1	1690000	1710000	1000000	1040000	64500	76300
Sample 2	1850000	1640000	940000	1050000	57300	52700
Sample 3	1750000	1610000	1010000	942000	64800	66100
Average	1763333	1653333	983333.3	1010667	62200	65033.33
STDEV	80829.04	51316.01	37859.39	59676.91	4246.175	11836.1
% CV	4.583877	3.103791	3.850107	5.904707	6.826648	18.20006
% Recovery		**106.7**		**97.3**		**95.6**
				Avg. % Recovery		**99.9**
				Avg. % CV		**7.1**

The mean recovery of nortriptyline from blood over the calibration range is 99.9 % with a % CV of 7.1 and meets the criteria for adequate recovery.

Table 7: Recovery for Citalopram

	High Concentration (1600 ng/mL)		Medium Concentration (800 ng/mL)		Low Concentration (45 ng/mL)	
	Test Sample: Peak Area	Theoretical Sample: Peak Area	Test Sample: Peak Area	Theoretical Sample: Peak Area	Test Sample: Peak Area	Theoretical Sample: Peak Area
Sample 1	1070000	680000	395000	500000	27700	32100
Sample 2	1030000	772000	491000	492000	25000	10000
Sample 3	875000	702000	442000	504000	22400	22000
Average	991666.7	718000	442666.7	498666.7	25033.3	21366.7
STDEV	102996.8	48041.7	48003.5	6110.1	2650.2	11063.6
% CV	10.4	6.7	10.8	1.2	10.6	51.8
% Recovery		**138.1**		**88.8**		**117.2**
				Avg. % Recovery		**114.7**
				Avg. % CV		**15.3**

The mean recovery of citalopram from blood over the calibration range is 114.7 % with a % CV of 15.3 and meets the criteria for adequate recovery. Overall, decent percentage recoveries and % CV values are seen for these three analytes and it is accepted that the extraction method described in section 2.7 is very efficient.

4.7 Matrix Effects

The matrix effects results for amitriptyline, nortriptyline and citalopram in blood is presented in Tables 8, 9 and 10 respectively and the overall % CV's of the slopes are calculated.

Table 8: Amitriptyline/ISTD peak area ratios

	High Conc. 1600 ng/mL Peak Area Ratio	Medium Conc. 800 ng/mL Peak Area Ratio	Low Conc. 45 ng/mL Peak Area Ratio	Area Ratio v Conc. Regression Slope
A	14.0	7.77	0.487	0.00869
B	12.6	6.63	0.526	0.00776
C	14.7	7.99	0.602	0.00905
D	14.2	6.75	0.523	0.00880
E	12.6	6.69	0.549	0.00775
F	11	6.83	0.779	0.00656
Average	13.2	7.11	0.578	0.00810
STDEV	0.964	0.602	0.105	0.000933
%CV	**7.3**	**8.5**	**18.3**	**11.5**

Table 9: Nortriptyline/ISTD peak area ratios

	High Conc. 1600 ng/mL Peak Area Ratio	Medium Conc. 800 ng/mL Peak Area Ratio	Low Conc. 45 ng/mL Peak Area Ratio	Area Ratio v Conc. Regression Slope
A	10	5.52	0.345	0.00620
B	10	5.80	0.344	0.00619
C	10	5.26	0.358	0.00619
D	9.84	4.92	0.319	0.00612
E	8.58	3.90	0.354	0.00523
F	8.19	4.58	0.385	0.00501
Average	9.44	4.99	0.351	0.00583
STDEV	0.752	0.688	0.0215	0.00053
%CV	**8**	**13.8**	**6.1**	**9.2**

Table 10: Citalopram/ISTD peak area ratios

	High Conc. 1600 ng/mL Peak Area Ratio	Medium Conc. 800 ng/mL Peak Area Ratio	Low Conc. 45 ng/mL Peak Area Ratio	Area Ratio v Conc. Regression Slope
A	3.78	1.61	0.108	0.00236
B	3.53	1.97	0.0905	0.00221
C	4.30	1.98	0.0892	0.00271
D	3.98	2.01	0.0793	0.00250
E	4.39	2	0.108	0.00275
F	3	1	0.201	0.00180
Average	3.83	1.76	0.113	0.00239
STDEV	0.52	0.404	0.0446	0.000350
%CV	**13.5**	**22.9**	**39.7**	**14.7**

Ideally, the slope variability (% CV) for the six different matrix sources (A - F) should not be more than 5% as this indicates that matrix effects do not adversely influence the precision of the assay. For amitriptyline, nortriptyline and citalopram, the %CV for the different blood sources was calculated to be 11.5%, 9.2% and 14.7% respectively. Although these values exceed the accepted 5%, it does not exceed the acceptance criteria by too much and for the purpose of this partial validation it provides a good indication of the effects of the components in the blood matrix other than the analytes. Further refinement of chromatography or of the extraction method could aid in the removal of interfering matrix components which may have an effect on the analyte and internal standard ionization. Matrix effects are an important factor in LC-MS/MS analyses and may become apparent when authentic samples are analysed (Castel, 2014).

4.8 Stability

Average peak areas of amitriptyline, nortriptyline and citalopram obtained from the analysis of duplicate quality controls at low, medium and high concentration levels in blood left at both 25 °Con the laboratory bench and at 4 °C in the refrigerator were compared to that of freshly prepared quality controls by plotting bar graphs as shown in Figure 4 below.

It can be seen that the peak areas of the analytes found from freshly prepared quality controls are in every case the highest as it represents the analytes at their most stable condition. Table 11 indicates the degree to which the stabilities differ at the different temperatures.

Table 11: Percentage difference values in average peak heights calculated for the analytes in each QC level left at 4 °C in the refrigerator and at 25 °C on the benchtop for one week compared to that of fresh samples, taking the average peak height of fresh analytes to be 100%

QC level and Concentration	Amitriptyline		Nortriptyline		Citalopram	
	Bench	Fridge	Bench	Fridge	Bench	Fridge
L QC: 45 ng/mL	87.7	78.2	28.6	22.1	0.4	-10.4
M QC:800 ng/mL	51.3	47.7	20.4	10.4	13.8	6.1
H QC: 1600 ng/mL	26.2	17.4	17.1	5.8	17.9	6.5

Storage at temperatures of 25 °C and 4 °C after one week does have an effect on the stability of the analytes as their peak areas are lower which is also indicated by lower bar graphs in Figure 4. High percentage difference values (greater than 15%) as seen in Table 11 give an approximate indication of whole blood instability (Castel, 2014). This is more evident for the analyte amitriptyline, especially for lower concentrations of the analyte as can be seen in the bar graphs for the low QC (45 ng/mL) and medium QC (800 ng/mL). It is also seen in Table 11 with high percentage differences from the fresh samples of 87.7% and 78.2% respectively. It is observed overall that there is a smaller difference in peak heights the more concentrated the samples are. Highly concentrated samples could thus have a lower risk of degrading at a fast pace.

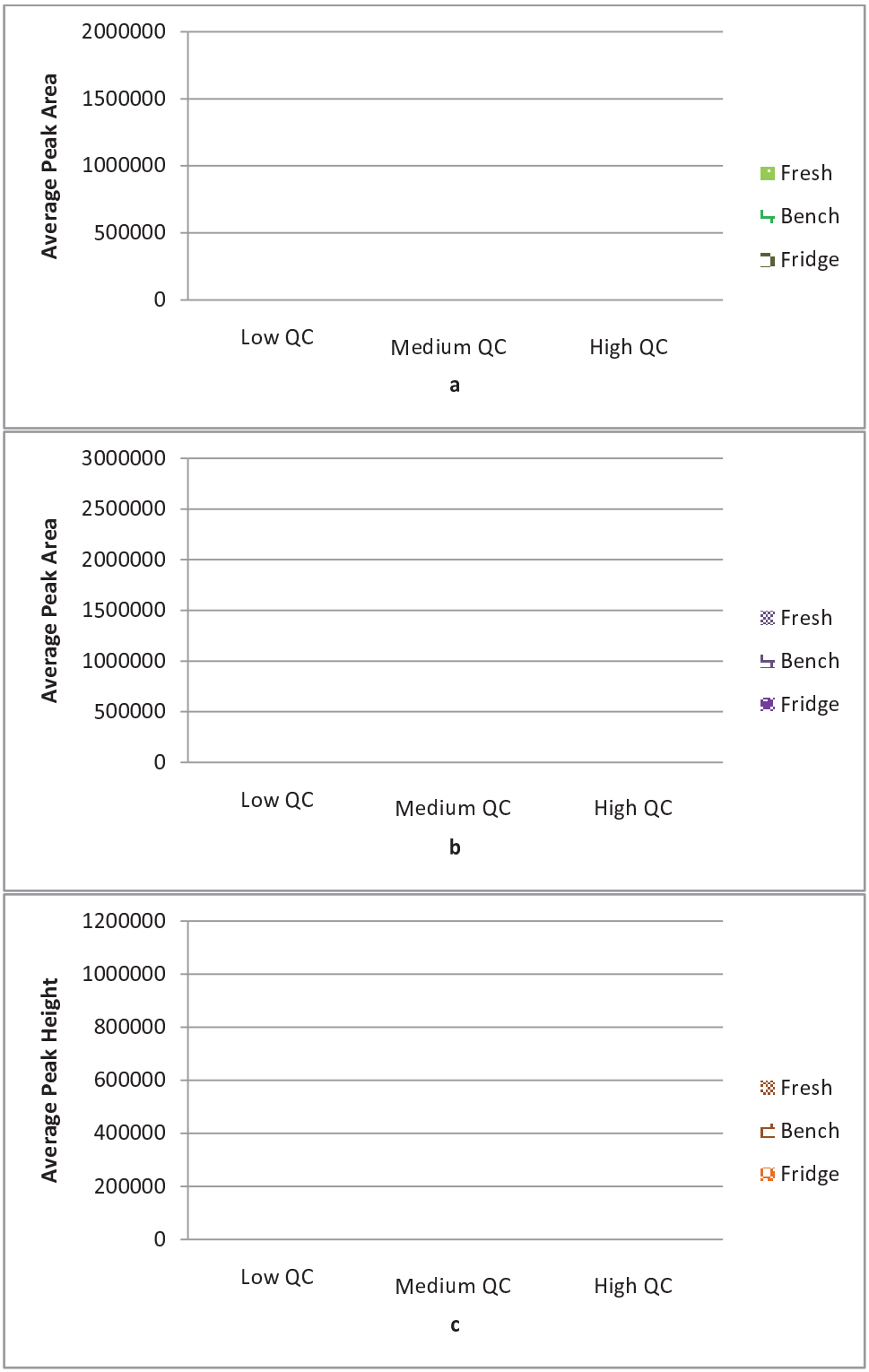


Figure 5: Bar graphs showing a comparison of the average peak areas for low, medium and high qualitycontrols (QC) freshly prepared or prepared after one week on the bench at room temperature (~ 25 °C)or in the fridge (4 °C). These graphs show the stability of the analytes amitriptyline (a), nortriptyline (b) and citalopram (c) under these conditions

In general a trend is observed where the peak areas are found to be the lowest for analytes left on the benchtop, compared to analytes stored in the refrigerator, which was the highest temperature out of the three temperatures that were tested. Stability of the analytes thus seems to be indirectly proportional to an increase in temperature and thus the lower the storage temperature of samples, the more stable the analytes will be. Furthermore it can be speculated that the longer samples are exposed to slightly elevated temperatures, the greater the decrease in stability of analytes will be. As stated, samples were left for only one week on the benchtop and the refrigerator and further test could be performed to investigate the effect on stability when left at higher temperatures for longer periods of time. Samples to be tested by the developed LC-MS/MS method should therefore preferably be stored at - 80 °C when it is not immediately analysed in order to test stable analytes and thus to obtain the most accurate results.

5. Conclusion

Extraction and HPLC-MS/MS methods were developed for the detection and quantification of the 11 target analytes in blood, serum and urine. Calibration curves for all matrices were found to be linear over the range tested with r^2 values greater than 0.95. Overall accuracy and precision results over three validation batches show that the method provides sufficient accuracy and precision over the entire range (15.6 – 2000 ng/mL) for all analytes in all three matrices – except for oxazepam in blood. This is based on analyte/internal standard peak area ratios with a quadratic calibration curve (weighted by 1/x concentration). In cases where acceptance criteria were not met, the %Accuracy values were not out by more than 8%.

Five different authentic samples were analysed using the methods developed in this study, including both clinical and post-mortem samples and samples from each of the three different biological matrices. The results obtained confirmed the use of the developed method for both the detection and quantification of the target drugs. Quantification results can subsequently provide interpretation and insight into the repercussions of the drug usage in clinical and forensic situations, especially if some information is known about the history surrounding the drug usage.

Partial validation was achieved for the method in the blood matrix. No remarkable carryover effects was recognised in chromatograms of double blank samples injected directly after a standard sample at the highest concentration level of the calibration curve. Whilstinvestigating sensitivity as a validation parameter, it was found that the instrument was sensitive enough to detect the level of the LLOQ concentration at 15.6 ng/mL.The extraction recovery of the method for the three antidepressants was found to be very efficient with consistent %Recovery values between concentration levels and the percentage coefficient of variation for mean measured recoveries not exceeding 15%, thus succeeding acceptance criteria.

Endogenous matrix components were found to not have an enormous effect on the reproducibility of the method when human blood originating from six different sources was analysed. The %CV values for the matrix experiment were found to be slightly above the accepted 5% and work could be done to reduce the effect of matrix constituents. Amitriptyline, nortriptyline and citalopram was found to be the less stable when stored in the refrigerator at 4 °C and the least stable when stored on the benchtop at 25 °C or one week. Samples to be tested with this method should therefore ideally be stored at - 80 °C for a limited time if not tested immediately after collection.

In summary, partial validation experiments reveal that the developed LC-MS/MS method is potentially suited for the analysis of the three antidepressants namely in blood. Future work should entail performing a full validation of the current procedure and analysing results for all 11 compounds.

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Role of Co-author

Professor Peter Smith

Gave administrative assistance and aided in the development of the study design and practical focus of the project.

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AUTHOR INFORMATION PACK

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Appendices

A: Blood Accuracy and Precision Validation Results

Here follows a list for the series of results that is presented for the Blood Accuracy and Precision Results. It can be seen that the following sequence of results is presented for each analyte:

- A representative calibration curve (including r^2 values)
- Calibration standard accuracy and precision results
- Summary of intra-validation quality control standards

} Presented for validation 1, 2 and 3 for each analyte

- Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3
- Overall Quality Control Accuracy and Precision Estimation

<u>Nortriptyline</u>: Blood Validation 1, Day1

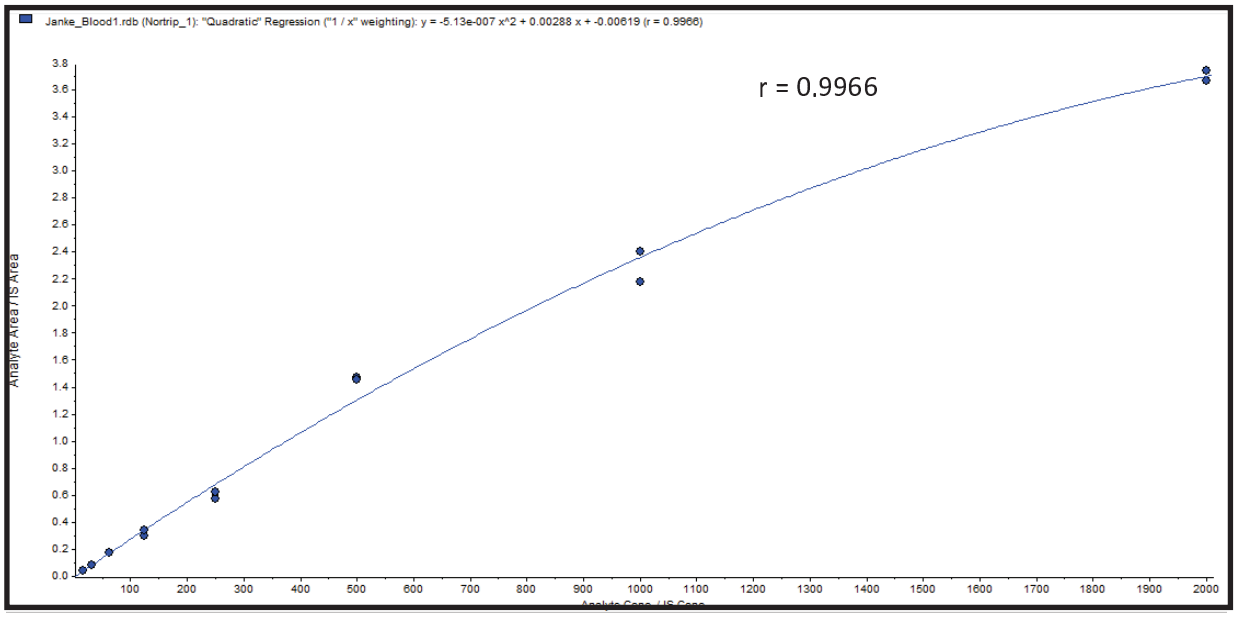


Figure A 1. Representative calibration curve for Nortriptyline: Validation 1, Day 1.

Table A 1.1. Nortriptyline Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.9	0.32	1.9	108.1
31.3	S7	2 of 2	30.4	0.09	0.3	97.1
62.5	S6	2 of 2	65	0.09	0.1	104
125	S5	2 of 2	116	12.3	10.5	93.1
250	S4	2 of 2	218	12.6	5.8	87.3
500	S3	2 of 2	569	4.42	0.8	113.8
1000	S2	2 of 2	966	83.2	8.6	96.6
2000	S1	2 of 2	2010	65.3	3.3	100.3

Table A 1.2. Summary of Nortriptyline intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	16.8	2.56	15.3	107.5
45	L QC	6 of 6	37.6	5.29	14.1	83.5
800	M QC	6 of 6	742	29.8	4	92.8
1600	H QC	6 of 6	1360	999	73.6	84.8

Nortriptyline: Blood Validation 2, Day 2

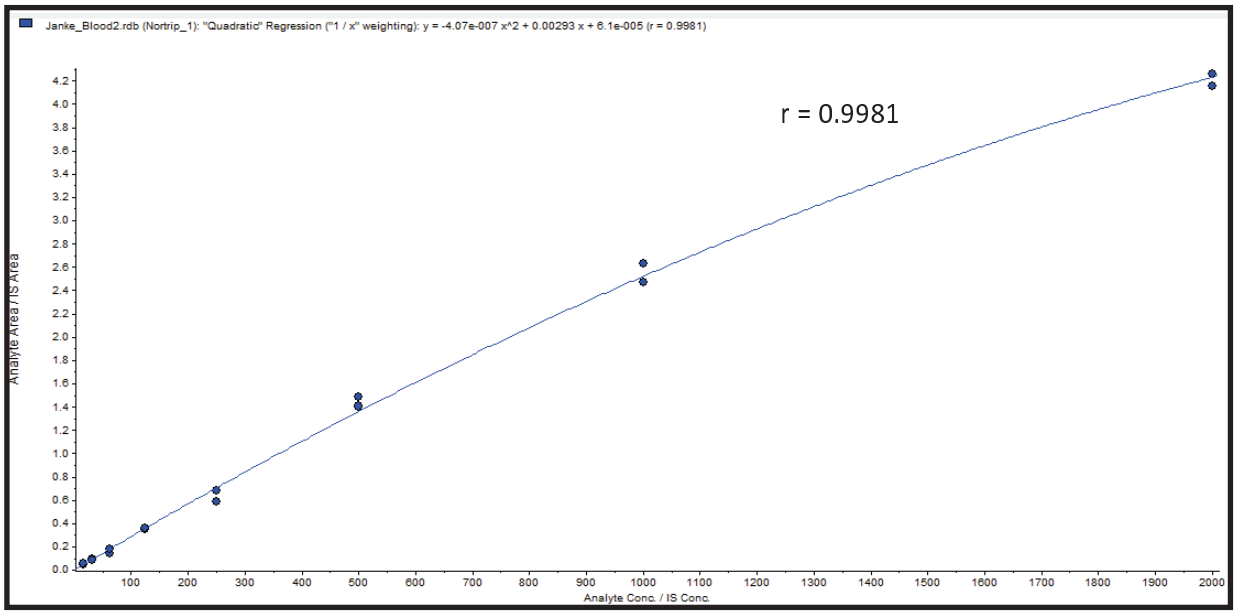


Figure A 2. Representative calibration curve for Nortriptyline: Validation 2, Day 2.

Table A 2.1. Nortriptyline Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	17.9	2.36	13.2	114.8
31.3	S7	2 of 2	31.2	0.97	3.1	99.7
62.5	S6	2 of 2	55.6	9.69	17.4	88.9
125	S5	2 of 2	125	2.52	2	100
250	S4	2 of 2	223	26	11.6	89.4
500	S3	2 of 2	534	22.8	4.3	106.7
1000	S2	2 of 2	1010	54.3	5.4	101.3
2000	S1	2 of 2	1980	57.7	2.9	99.1

Table A 2.2. Summary of Nortriptyline intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	Accuracy
15.6	LLOQ	6 of 6	16.6	3.16	19	106.7
45	L QC	6 of 6	43	5.87	13.6	95.6
800	M QC	6 of 6	809	74.4	9.2	101.1
1600	H QC	6 of 6	1410	662	46.9	88.4

Nortriptyline: Blood Validation 3, Day 3

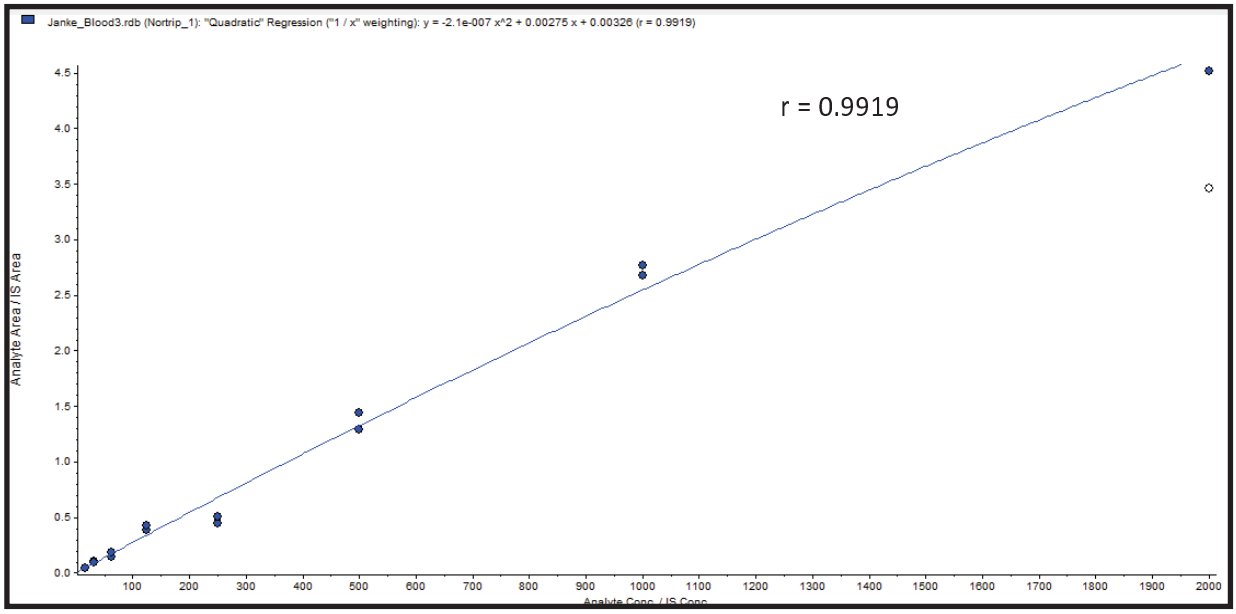


Figure A 3. Representative calibration curve for Nortriptyline: Validation 3, Day 3.

Table A 3.1. Nortriptyline Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	14.9	0.25	1.7	95.8
31.3	S7	2 of 2	35.4	3.14	8.9	113
62.5	S6	2 of 2	59.2	11.2	19	94.7
125	S5	2 of 2	147	9.82	6.7	117.6
250	S4	2 of 2	176	15.5	8.8	70.3
500	S3	2 of 2	515	43.7	8.5	103
1000	S2	2 of 2	1080	29.3	2.7	107.6
2000	S1	1 of 2	14.9	0.25	1.7	95.8

Table A 3.2. Summary of Nortriptyline intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	19.1	3	15.7	122.2
45	L QC	6 of 6	49.2	4.59	9.3	109.4
800	M QC	6 of 6	823	49.5	6	102.9
1600	H QC	6 of 6	1660	208	12.5	104

Table A 4.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	16.6	0.98	5.6	106.3
31.3	S7	6 of 6	32.3	1.4	4.1	103.3
62.5	S6	6 of 6	59.9	7.01	12.2	95.9
125	S5	6 of 6	129	8.2	6.4	103.6
250	S4	6 of 6	206	18	8.7	82.3
500	S3	6 of 6	539	23.7	4.5	107.8
1000	S2	6 of 6	1020	55.6	5.6	101.8
2000	S1	5 of 6	1970	61.5	3.1	98.5

Table A 4.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	18 of 18	17.5	2.91	16.7	112.1
45	L QC	18 of 18	43.3	5.25	12.3	96.2
800	M QC	18 of 18	791	51.2	6.4	98.9
1600	H QC	18 of 18	1480	623	44.3	92.4

<u>Amitriptyline</u>: Blood Validation 1, Day1

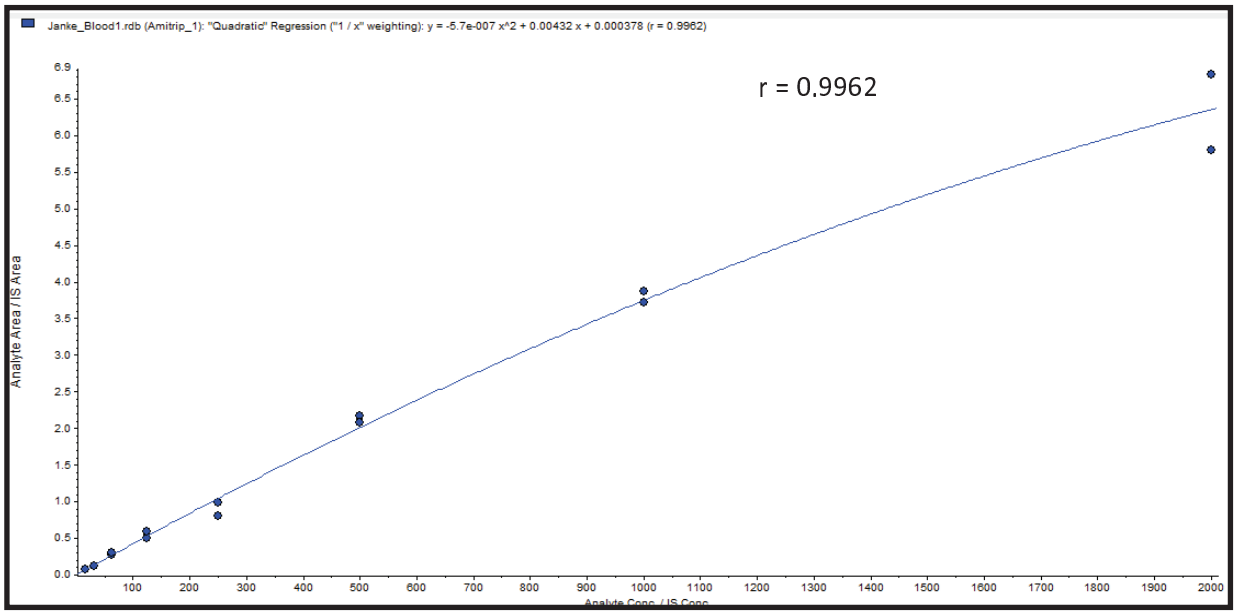


Figure A 4. Representative calibration curve for Amitriptyline: Validation 1, Day 1.

Table A 5.1. Amitriptyline Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	17.2	0.18	1	110.2
31.3	S7	2 of 2	27.8	0.01	0	88.8
62.5	S6	2 of 2	66.2	5.51	8.3	106
125	S5	2 of 2	129	15.8	12.3	102.9
250	S4	2 of 2	214	30.1	14.1	85.4
500	S3	2 of 2	529	16.1	3	105.9
1000	S2	2 of 2	1020	34.4	3.4	101.7
2000	S1	2 of 2	2000	357	17.9	100

Table A 5.2. Summary of Amitriptyline intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	15.3	2.37	15.5	97.8
45	L QC	6 of 6	40.7	6.61	16.2	90.6
800	M QC	6 of 6	757	52.2	6.9	94.6
1600	H QC	6 of 6	1300	832	63.9	81.3

<u>Amitriptyline</u>: Blood Validation 2, Day 2

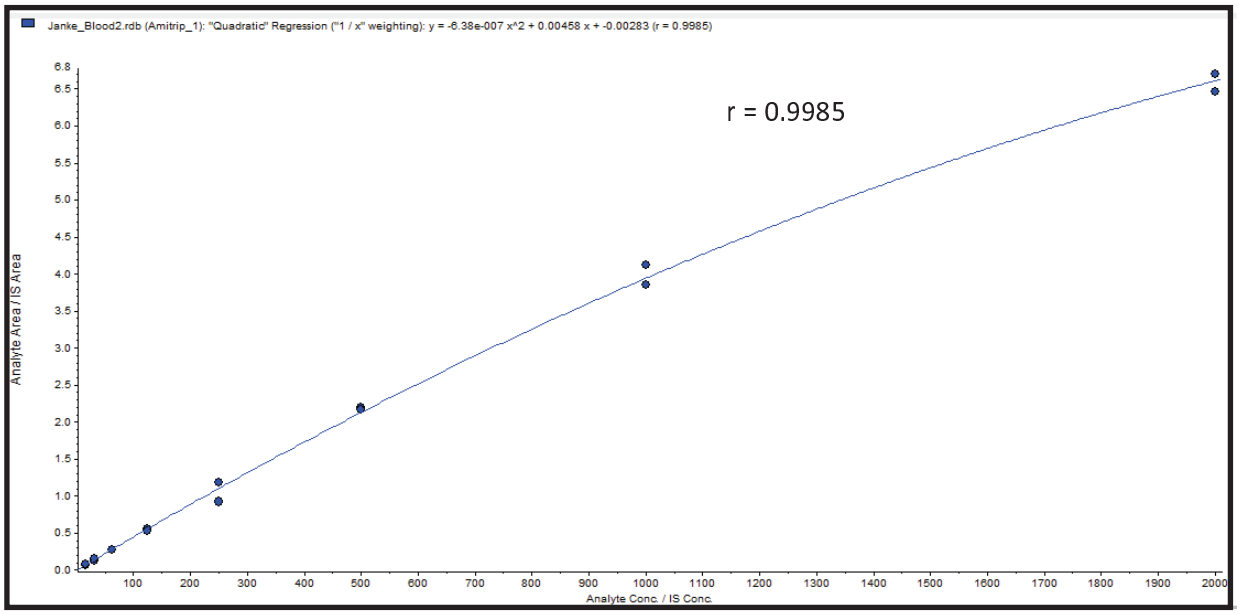


Figure A 5. Representative calibration curve for Amitriptyline: Validation 2, Day 2.

Table A 6.1. Amitriptyline Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	17.9	2.36	13.2	114.8
31.3	S7	2 of 2	31.2	0.97	3.1	99.7
62.5	S6	2 of 2	55.6	9.69	17.4	88.9
125	S5	2 of 2	125	2.52	2	100
250	S4	2 of 2	223	26	11.6	89.4
500	S3	2 of 2	534	22.8	4.3	106.7
1000	S2	2 of 2	1010	54.3	5.4	101.3
2000	S1	2 of 2	1980	57.7	2.9	99.1

Table A 6.2. Summary of Amitriptyline intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	16.6	3.16	19	106.7
45	L QC	6 of 6	43	5.87	13.6	95.6
800	M QC	6 of 6	809	74.4	9.2	101.1
1600	H QC	6 of 6	1410	662	46.9	88.4

<u>Amitriptyline</u>: Blood Validation 3, Day3

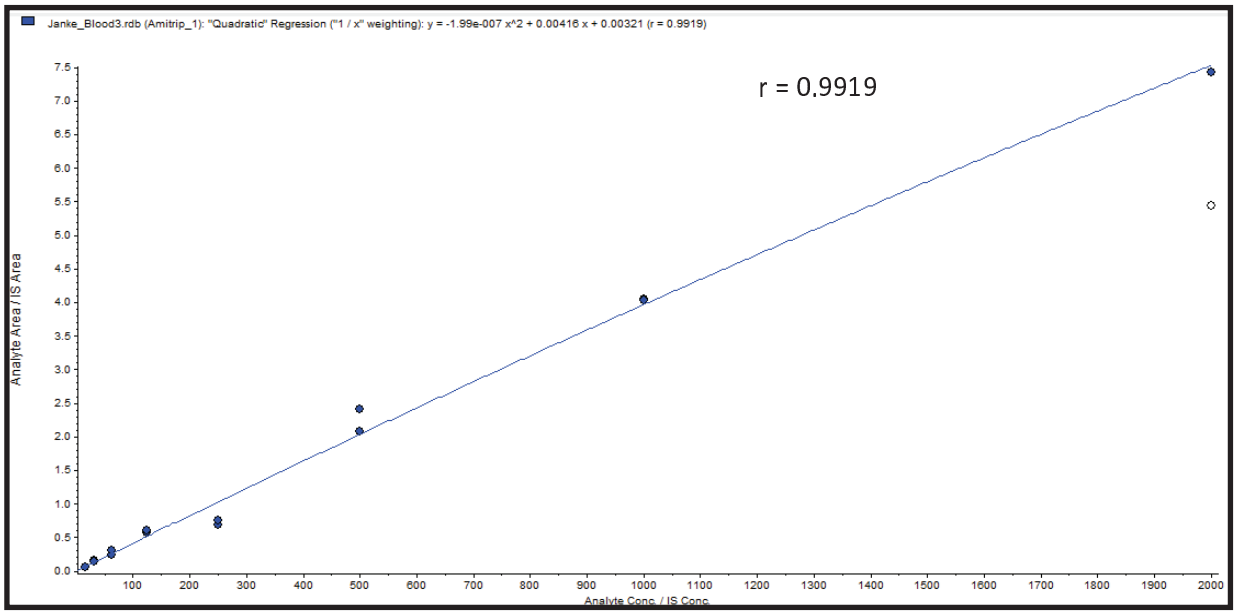


Figure A 6. Representative calibration curve for Amitriptyline: Validation 3, Day 3.

Table A 7.1. Amitriptyline Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.5	1.08	8	86.8
31.3	S7	2 of 2	36.2	2.21	6.1	115.7
62.5	S6	2 of 2	64.9	13.7	21	103.8
125	S5	2 of 2	141	3.45	2.4	112.9
250	S4	2 of 2	173	11.9	6.9	69
500	S3	2 of 2	554	60.3	10.9	110.8
1000	S2	2 of 2	1020	3.19	0.3	101.8
2000	S1	1 of 2	1970	N/A	N/A	98.5

Table A 7.2. Summary of Amitriptyline intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	Accuracy
15.6	LLOQ	6 of 6	16.4	1.48	9.1	104.9
45	L QC	6 of 6	45.1	4.94	11	100.2
800	M QC	6 of 6	792	39.5	5	99
1600	H QC	6 of 6	1580	183	11.5	99.1

TableA8.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	16.2	1.21	7.4	103.9
31.3	S7	6 of 6	31.7	1.06	3.1	101.4
62.5	S6	6 of 6	62.2	9.62	15.6	99.6
125	S5	6 of 6	132	7.27	5.6	105.3
250	S4	6 of 6	203	22.6	10.9	81.3
500	S3	6 of 6	539	33.1	6.1	107.8
1000	S2	6 of 6	1020	30.6	3	101.6
2000	S1	5 of 6	1980	207	10.4	99.2

TableA8.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	18 of 18	16.1	2.34	14.5	103.1
45	L QC	18 of 18	43	5.81	13.6	95.5
800	M QC	18 of 18	786	55.4	7	98.3
1600	H QC	18 of 18	1430	559	40.8	89.6

Citalopram: Blood Validation 1, Day 1

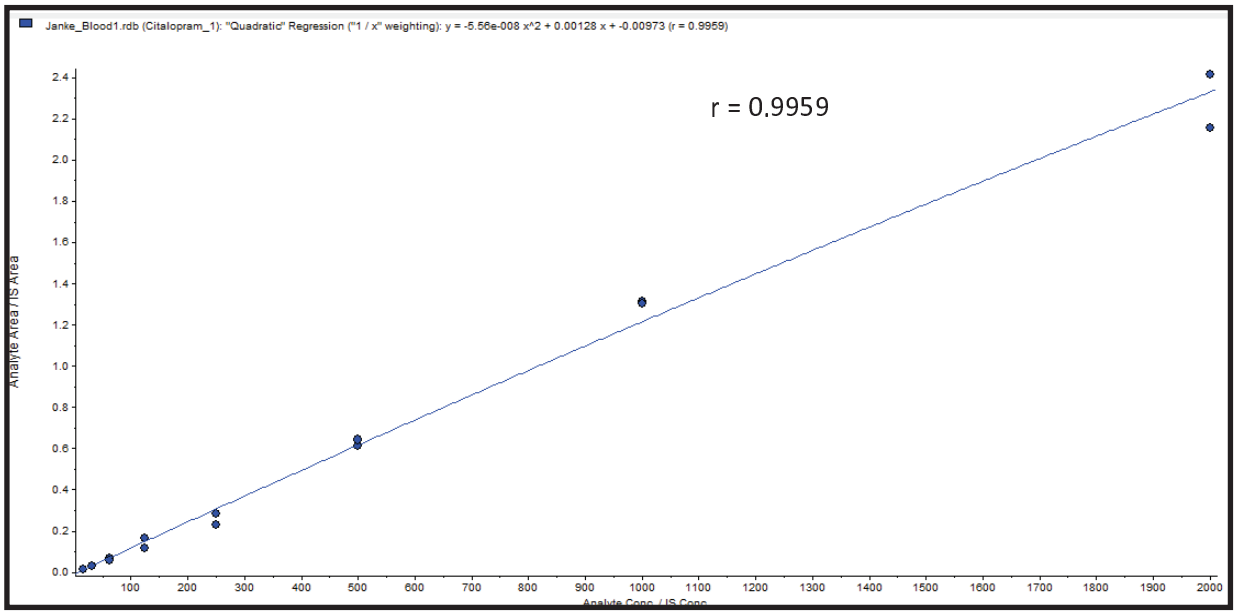


Figure A7. Representative calibration curve for Citalopram: Validation 1, Day 1.

Table A 9.1. Citalopram Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	19.2	1.54	8	123.3
31.3	S7	2 of 2	31.5	0.75	2.4	100.8
62.5	S6	2 of 2	56	7.25	12.9	89.6
125	S5	2 of 2	118	27.7	23.5	94.4
250	S4	2 of 2	210	32.2	15.3	84.1
500	S3	2 of 2	508	18.4	3.6	101.7
1000	S2	2 of 2	1080	7.74	0.7	108.2
2000	S1	2 of 2	1960	171	8.7	98

Table A 9.2. Summary of Citalopram intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	20.7	1.47	7.1	132.9
45	L QC	6 of 6	43.7	4.33	9.9	97.2
800	M QC	6 of 6	701	47.3	6.7	87.7
1600	H QC	6 of 6	1240	792	64	77.3

Citalopram: Blood Validation 2, Day2

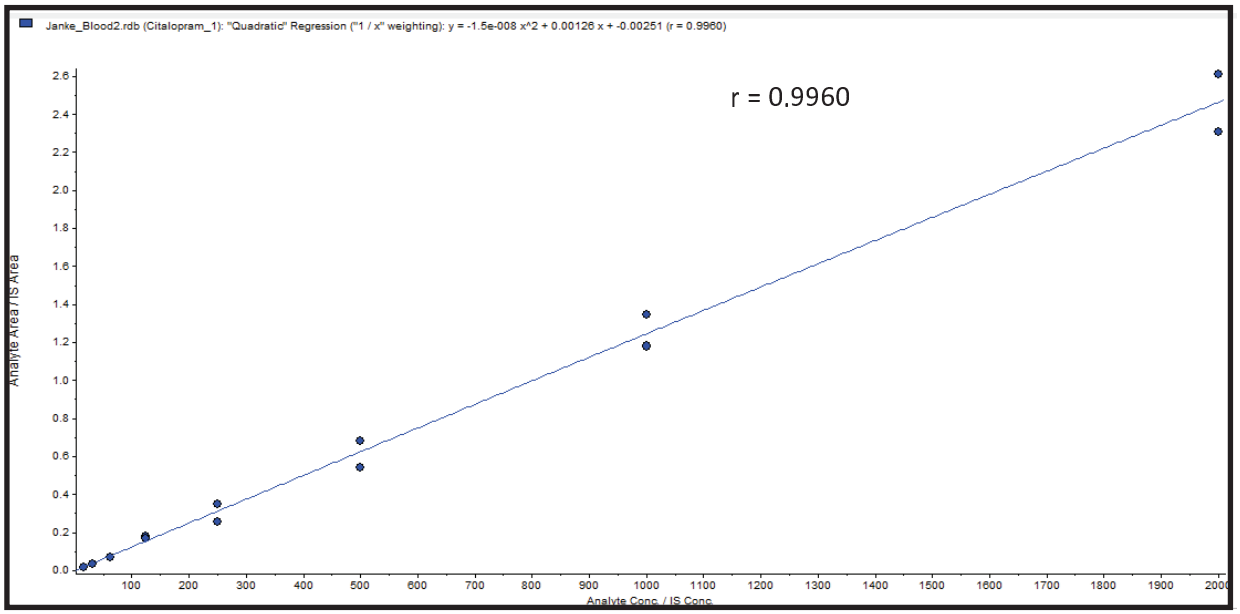


Figure A 8. Representative calibration curve for Citalopram: Validation 2, Day 2.

Table A 10.1. Citalopram Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	17.9	2.36	13.2	114.8
31.3	S7	2 of 2	31.2	0.97	3.1	99.7
62.5	S6	2 of 2	55.6	9.69	17.4	88.9
125	S5	2 of 2	125	2.52	2	100
250	S4	2 of 2	223	26	11.6	89.4
500	S3	2 of 2	534	22.8	4.3	106.7
1000	S2	2 of 2	1010	54.3	5.4	101.3
2000	S1	2 of 2	1980	57.7	2.9	99.1

Table A 10.2. Summary of Citalopram intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	16.6	3.16	19	106.7
45	L QC	6 of 6	43	5.87	13.6	95.6
800	M QC	6 of 6	809	74.4	9.2	101.1
1600	H QC	6 of 6	1410	662	46.9	88.4

Citalopram: Blood Validation 3, Day 3

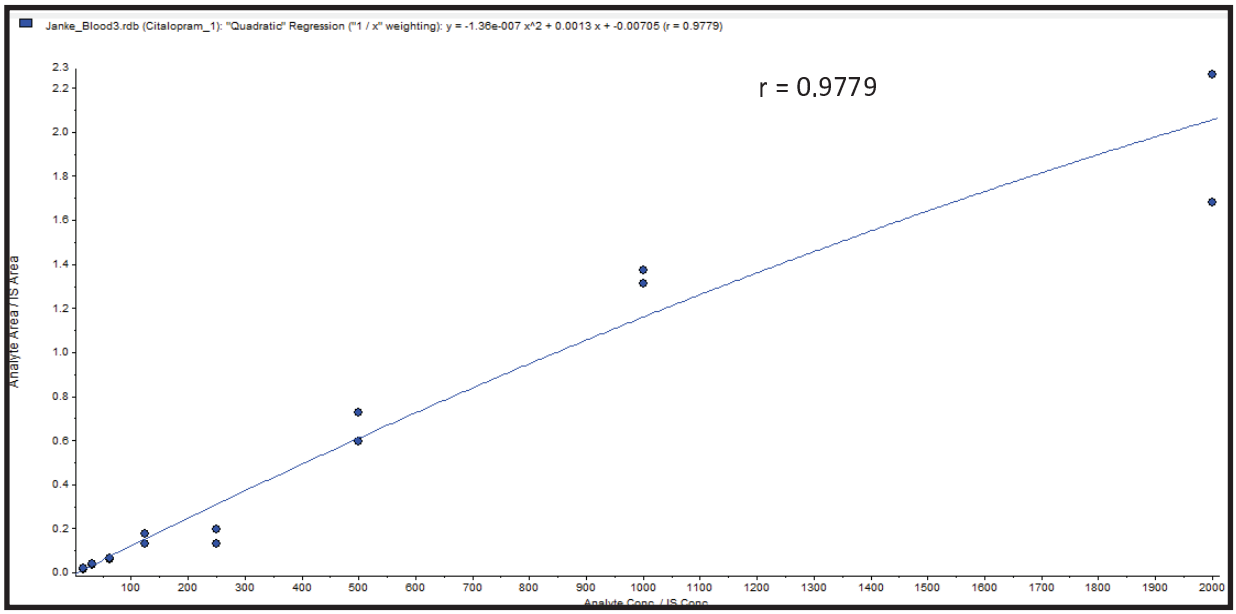


Figure A 9. Representative calibration curve for Citalopram: Validation 3, Day 3.

Table A 11.1. Citalopram Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	20	4.07	20.4	128
31.3	S7	2 of 2	35.2	3.71	10.5	112.4
62.5	S6	2 of 2	54.1	2.92	5.4	86.5
125	S5	2 of 2	125	25.6	20.6	99.6
250	S4	2 of 2	132	36.6	27.7	52.9
500	S3	2 of 2	545	79.6	14.6	109
1000	S2	2 of 2	1180	42.8	3.6	118
2000	S1	2 of 2	1910	526	27.5	95.7

Table A 11.2. Summary of Citalopram intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	20.9	2.33	11.1	134.1
45	L QC	6 of 6	46.7	8.03	17.2	103.8
800	M QC	6 of 6	849	141	16.6	106.1
1600	H QC	6 of 6	2260	302	13.4	141

Table A 12.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	19	2.65	13.8	122
31.3	S7	6 of 6	32.7	1.81	5.3	104.3
62.5	S6	6 of 6	55.2	6.62	11.9	88.3
125	S5	6 of 6	123	18.6	15.4	98
250	S4	6 of 6	189	31.6	18.2	75.5
500	S3	6 of 6	529	40.3	7.5	105.8
1000	S2	6 of 6	1090	34.9	3.2	109.2
2000	S1	6 of 6	1950	252	13	97.6

Table A 12.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	18 of 18	19.4	2.32	12.4	124.6
45	L QC	18 of 18	44.5	6.07	13.6	98.9
800	M QC	18 of 18	786	87.6	10.9	98.3
1600	H QC	18 of 18	1640	585	41.4	102.2

Alprazolam: Blood Validation 1, Day1

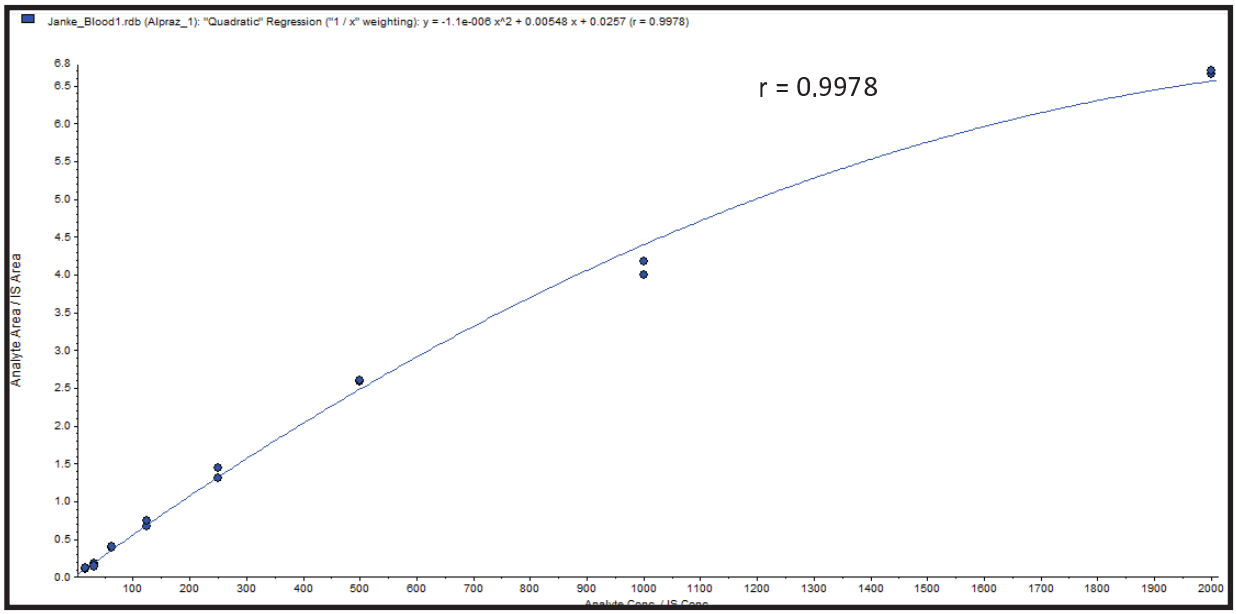


Figure A 10. Representative calibration curve for Alprazolam: Validation 1, Day 1.

Table A 13.1. Alprazolam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.5	1.89	12.2	99.5
31.3	S7	2 of 2	26.1	5.49	21	83.5
62.5	S6	2 of 2	69.5	3.33	4.8	111.2
125	S5	2 of 2	128	8.67	6.8	102.6
250	S4	2 of 2	261	20.1	7.7	104.3
500	S3	2 of 2	526	2.43	0.5	105.2
1000	S2	2 of 2	909	34.5	3.8	90.9
2000	S1	2 of 2	2130	40.3	1.9	106.5

Table A 13.2. Summary of Alprazolam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	Accuracy
15.6	LLOQ	6 of 6	14.4	1.97	13.7	92.2
45	L QC	6 of 6	41.7	5.79	13.9	92.6
800	M QC	6 of 6	723	34.1	4.7	90.4
1600	H QC	6 of 6	1210	877	72.7	75.3

Alprazolam: Blood Validation 2, Day 2

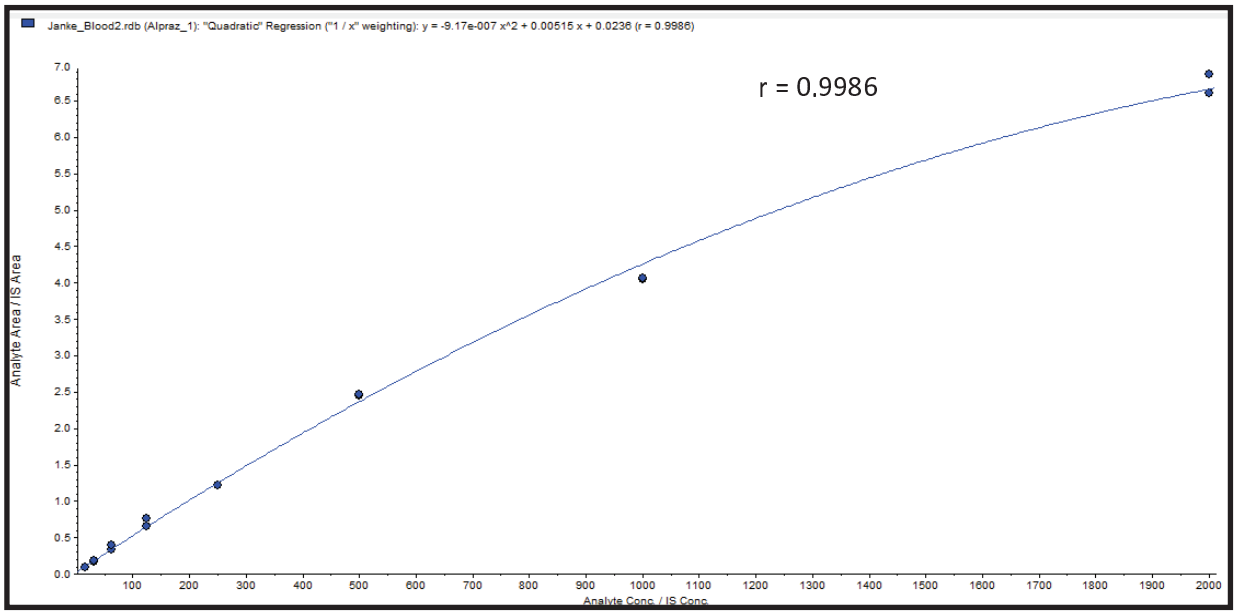


Figure A 11. Representative calibration curve for Alprazolam: Validation 2, Day 2.

Table A 14.1. Alprazolam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.8	0.66	4.8	88.4
31.3	S7	2 of 2	30.4	2.52	8.3	97.2
62.5	S6	2 of 2	67.9	8.98	13.2	108.6
125	S5	2 of 2	136	15.8	11.7	108.4
250	S4	2 of 2	243	0.18	0.1	97.3
500	S3	2 of 2	520	2.58	0.5	104.1
1000	S2	2 of 2	942	2.83	0.3	94.2
2000	S1	2 of 2	2060	133	6.5	102.9

Table A 14.2. Summary of Alprazolam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	16.9	12.3	72.6	108.3
45	L QC	6 of 6	40.3	5.99	14.9	89.6
800	M QC	6 of 6	757	76.1	10.1	94.6
1600	H QC	6 of 6	1440	681	47.3	90.1

Alprazolam: Blood Validation 3, Day3

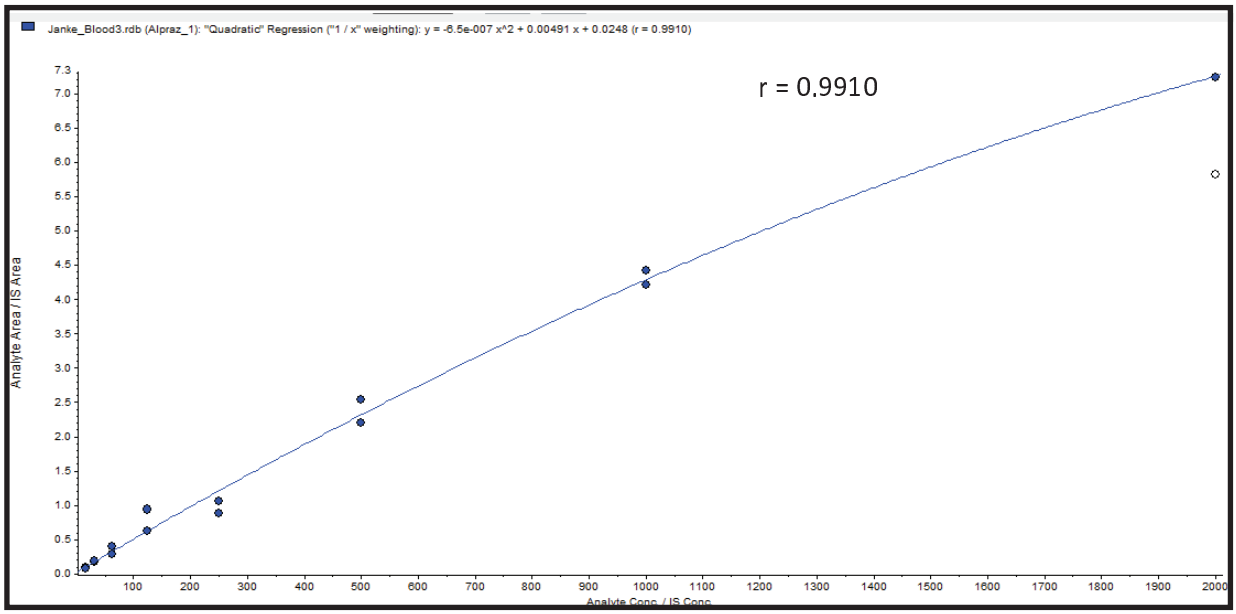


Figure A 12. Representative calibration curve for Alprazolam: Validation 3, Day 3.

Table A 15.1. Alprazolam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	12.2	2.02	16.6	78.2
31.3	S7	2 of 2	34	2.63	7.7	108.6
62.5	S6	2 of 2	65.5	14.8	22.6	104.8
125	S5	2 of 2	158	47.4	30.1	126.1
250	S4	2 of 2	199	27.6	13.9	79.6
500	S3	2 of 2	512	57.9	11.3	102.5
1000	S2	2 of 2	1010	42.2	4.2	100.8
2000	S1	1 of 2	12.2	2.02	16.6	78.2

Table A 15.2. Summary of Alprazolam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	15.7	2.7	17.2	100.7
45	L QC	6 of 6	45	4.78	10.6	100
800	M QC	6 of 6	788	51.2	6.5	98.5
1600	H QC	6 of 6	1580	225	14.2	98.6

Table A 16.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	13.8	1.53	11.2	88.7
31.3	S7	6 of 6	30.2	3.55	12.3	96.4
62.5	S6	6 of 6	67.6	9.04	13.6	108.2
125	S5	6 of 6	140	24	16.2	112.4
250	S4	6 of 6	234	15.9	7.2	93.7
500	S3	6 of 6	519	21	4.1	103.9
1000	S2	6 of 6	953	26.5	2.8	95.3
2000	S1	5 of 6	2060	86.7	4.2	103

Table A 16.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	18 of 18	15.7	5.64	34.5	100.4
45	L QC	18 of 18	42.3	5.52	13.1	94.1
800	M QC	18 of 18	756	53.8	7.1	94.5
1600	H QC	18 of 18	1410	594	44.7	88

<u>Clonazepam</u>: Blood Validation 1, Day1

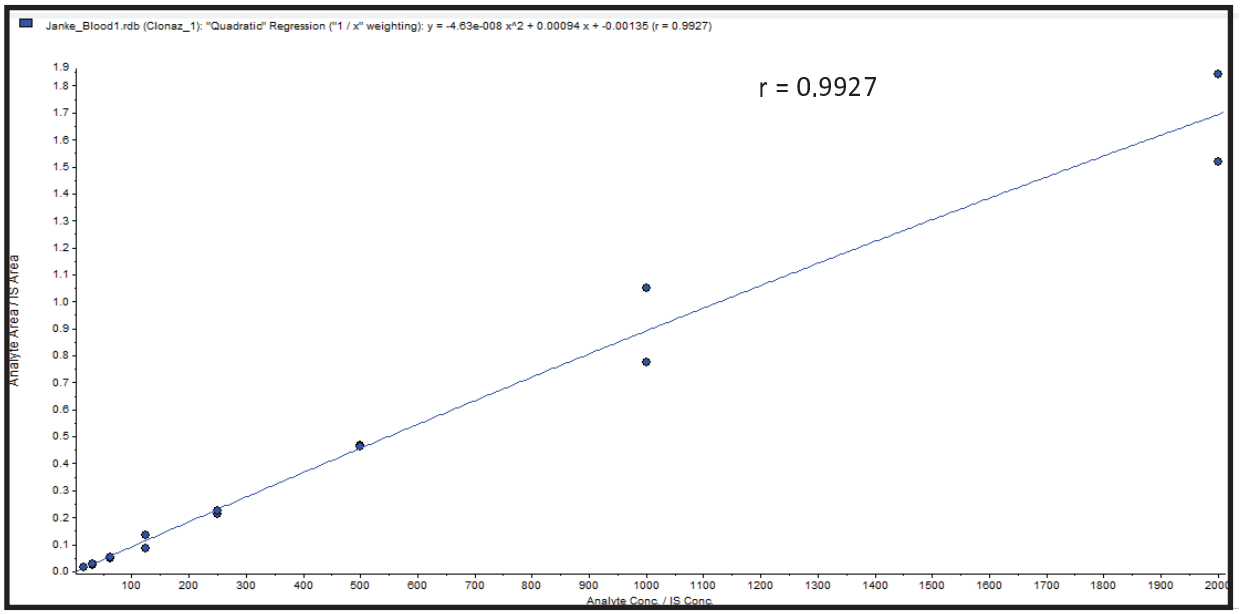


Figure A 13. Representative calibration curve for Clonazepam: Validation 1, Day 1.

Table A 17.1. Clonazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	18.4	0.4	2.2	118.1
31.3	S7	2 of 2	29.7	1.19	4	94.8
62.5	S6	2 of 2	57.7	2.63	4.6	92.3
125	S5	2 of 2	120	37.9	31.7	95.7
250	S4	2 of 2	238	8.28	3.5	95.1
500	S3	2 of 2	510	3.99	0.8	102
1000	S2	2 of 2	1030	230	22.4	102.7
2000	S1	2 of 2	1990	302	15.2	99.4

Table A 17.2. Summary of Clonazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	15.6	2.5	16	100
45	L QC	6 of 6	40.7	3.92	9.6	90.4
800	M QC	6 of 6	777	53.3	6.9	97.2
1600	H QC	6 of 6	1280	818	64	79.8

Clonazepam: Blood Validation 2, Day 2

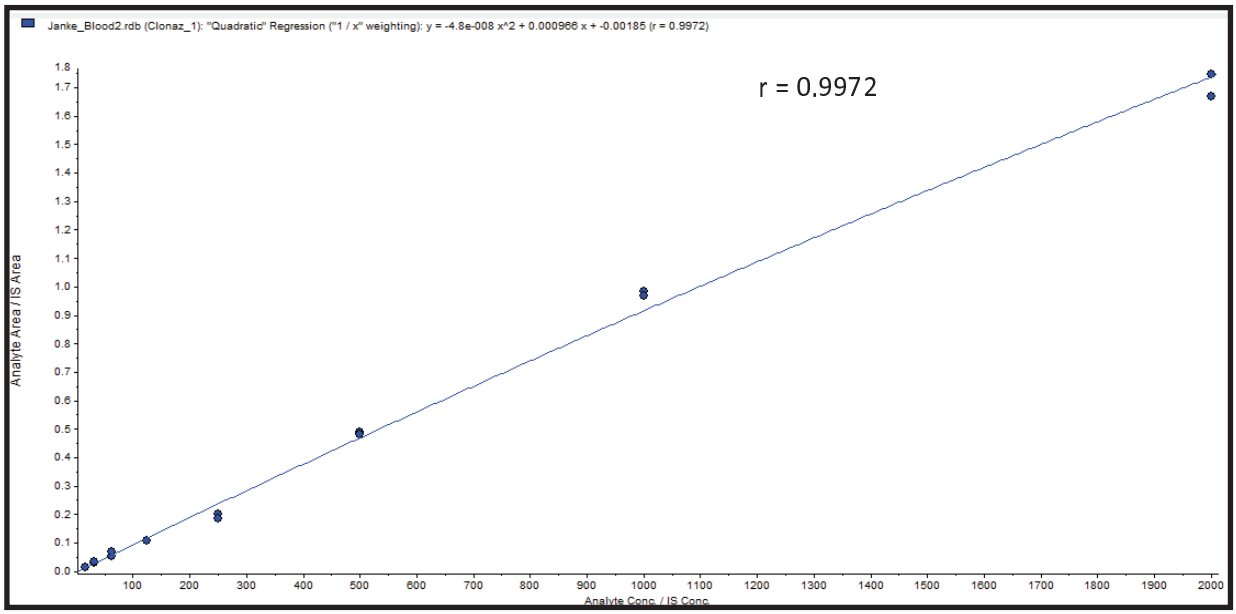


Figure A 14. Representative calibration curve for Clonazepam: Validation 2, Day 2.

Table A 18.1. Clonazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.6	0.39	2.4	106.1
31.3	S7	2 of 2	34.2	2.7	7.9	109.2
62.5	S6	2 of 2	64.4	11.4	17.7	103.1
125	S5	2 of 2	114	2.33	2	91.3
250	S4	2 of 2	204	11.1	5.4	81.5
500	S3	2 of 2	518	7.21	1.4	103.5
1000	S2	2 of 2	1070	14.4	1.3	107
2000	S1	2 of 2	1960	70.3	3.6	98.2

Table A 18.2. Summary of Clonazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	17.2	7.4	43.1	110.1
45	L QC	6 of 6	40.4	6.16	15.2	89.8
800	M QC	6 of 6	841	98.8	11.8	105.1
1600	H QC	6 of 6	1470	688	46.9	91.7

<u>Clonazepam</u>: Blood Validation 3, Day3

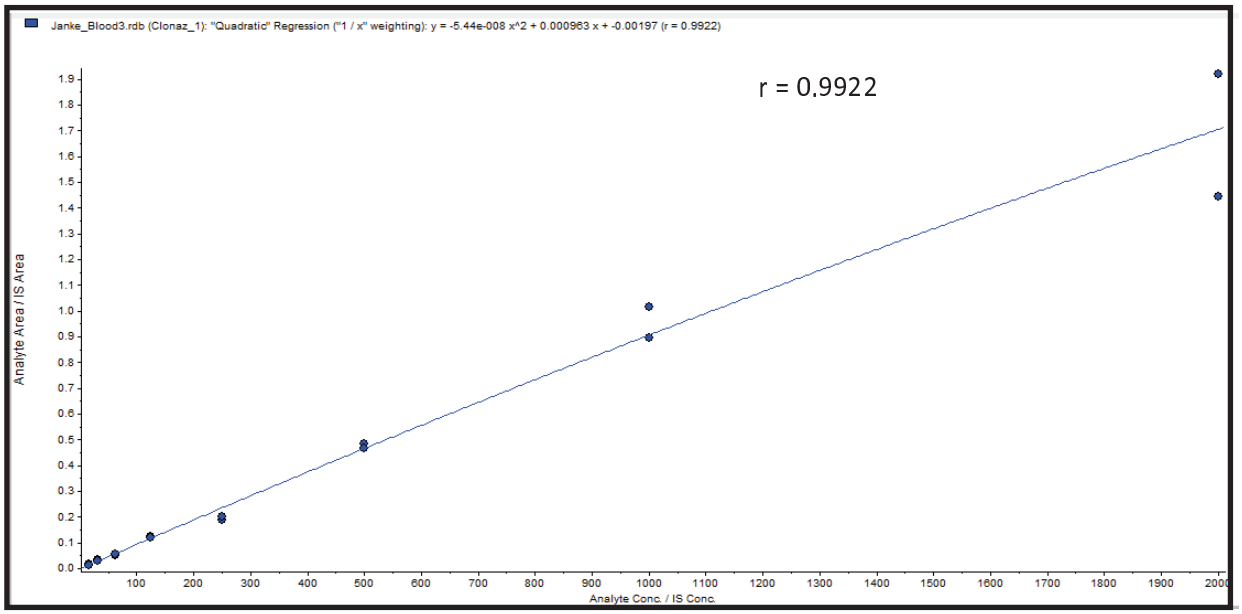


Figure A 15. Representative calibration curve for Clonazepam: Validation 3, Day 3.

Table A 19.1. Clonazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.7	3.1	18.6	106.8
31.3	S7	2 of 2	33.6	3.71	11.1	107.2
62.5	S6	2 of 2	58.2	1.67	2.9	93.1
125	S5	2 of 2	129	5.63	4.4	103.5
250	S4	2 of 2	207	10.4	5	82.8
500	S3	2 of 2	511	14.4	2.8	102.2
1000	S2	2 of 2	1060	98.8	9.3	105.9
2000	S1	2 of 2	1980	449	22.7	98.9

Table A 19.2. Summary of Clonazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	17.6	1.13	6.4	112.6
45	L QC	6 of 6	43.1	6.22	14.4	95.8
800	M QC	6 of 6	856	47.3	5.5	107.1
1600	H QC	6 of 6	1840	208	11.3	115.2

Table A 20.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	17.2	1.3	7.7	110.3
31.3	S7	6 of 6	32.5	2.54	7.7	103.7
62.5	S6	6 of 6	60.1	5.23	8.4	96.1
125	S5	6 of 6	121	15.3	12.7	96.8
250	S4	6 of 6	216	9.92	4.7	86.5
500	S3	6 of 6	513	8.53	1.7	102.6
1000	S2	6 of 6	1050	114	11	105.2
2000	S1	6 of 6	1980	274	13.8	98.8

Table A 20.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	18 of 18	16.8	3.68	21.8	107.6
45	L QC	18 of 18	41.4	5.43	13.1	92
800	M QC	18 of 18	825	66.5	8	103.1
1600	H QC	18 of 18	1530	571	40.7	95.6

<u>Diazepam</u>: Blood Validation 1, Day1

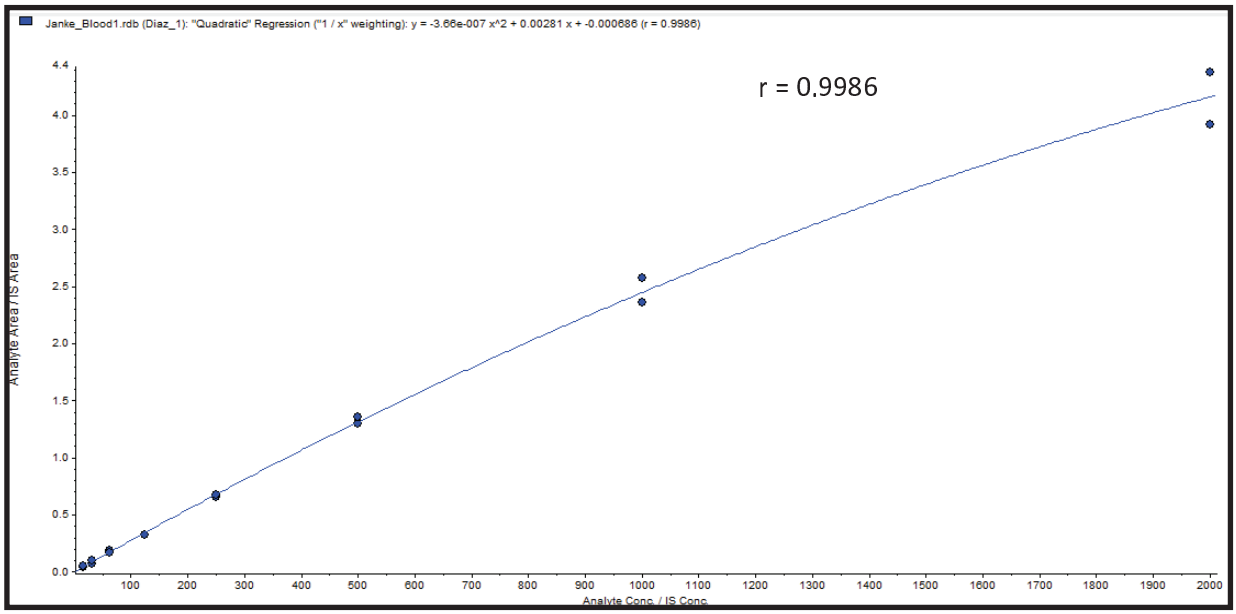


Figure A 16. Representative calibration curve for Diazepam: Validation 1, Day 1.

Table A 21.1. Diazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.1	1.69	10.5	103.4
31.3	S7	2 of 2	31	5.5	17.7	99.1
62.5	S6	2 of 2	64.2	3.7	5.8	102.7
125	S5	2 of 2	118	0.88	0.7	94.6
250	S4	2 of 2	245	5.2	2.1	98.1
500	S3	2 of 2	507	16.5	3.3	101.4
1000	S2	2 of 2	1010	74.5	7.4	101.1
2000	S1	2 of 2	2000	239	12	99.9

Table A 21.2. Summary of Diazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	15.6	1.95	12.5	99.9
45	L QC	6 of 6	38.8	1.44	3.7	86.2
800	M QC	6 of 6	733	54.1	7.4	91.7
1600	H QC	6 of 6	1280	807	63.2	79.8

<u>Diazepam</u>: Blood Validation 2, Day 2

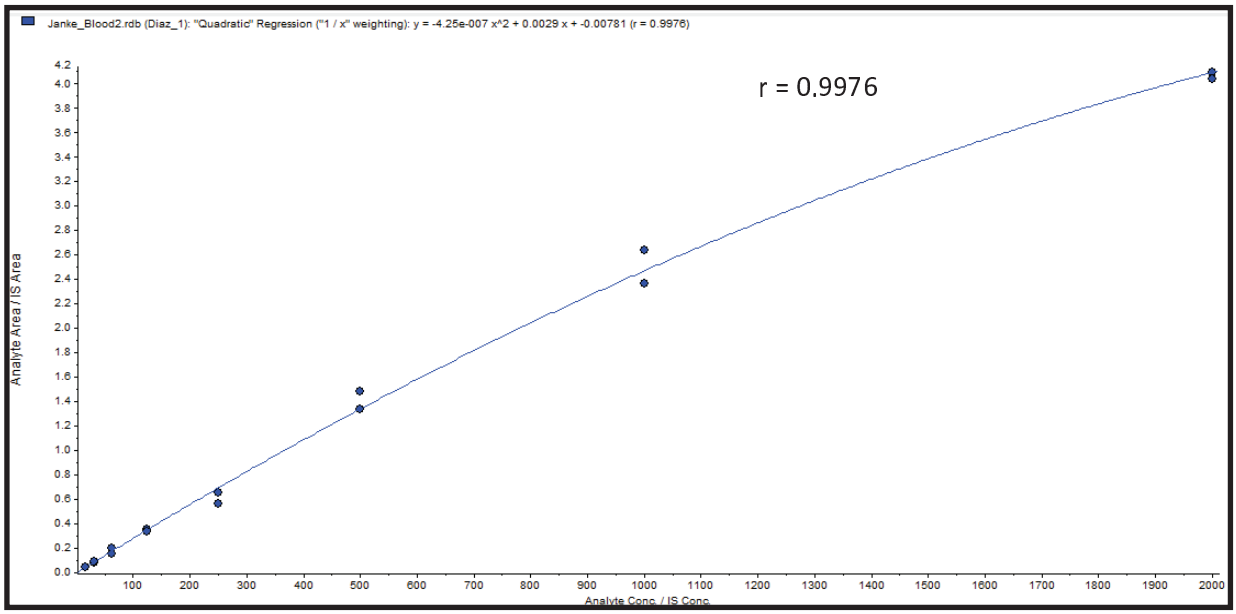


Figure A 17. Representative calibration curve for Diazepam: Validation 2, Day 2.

Table A22.1. Diazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	17	0.5	2.9	109.2
31.3	S7	2 of 2	30.3	2.52	8.3	96.9
62.5	S6	2 of 2	62.5	11.4	18.2	100
125	S5	2 of 2	124	3.96	3.2	99.2
250	S4	2 of 2	220	23.2	10.6	87.8
500	S3	2 of 2	530	42.7	8.1	106.1
1000	S2	2 of 2	1020	94.8	9.3	101.8
2000	S1	2 of 2	1980	31.9	1.6	98.9

Table A22.2. Summary of Diazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	19.6	7.72	39.5	125.4
45	L QC	6 of 6	39.7	2.6	6.5	88.2
800	M QC	6 of 6	781	87	11.1	97.6
1600	H QC	6 of 6	1460	681	46.6	91.3

<u>Diazepam</u>: Blood Validation 3, Day3

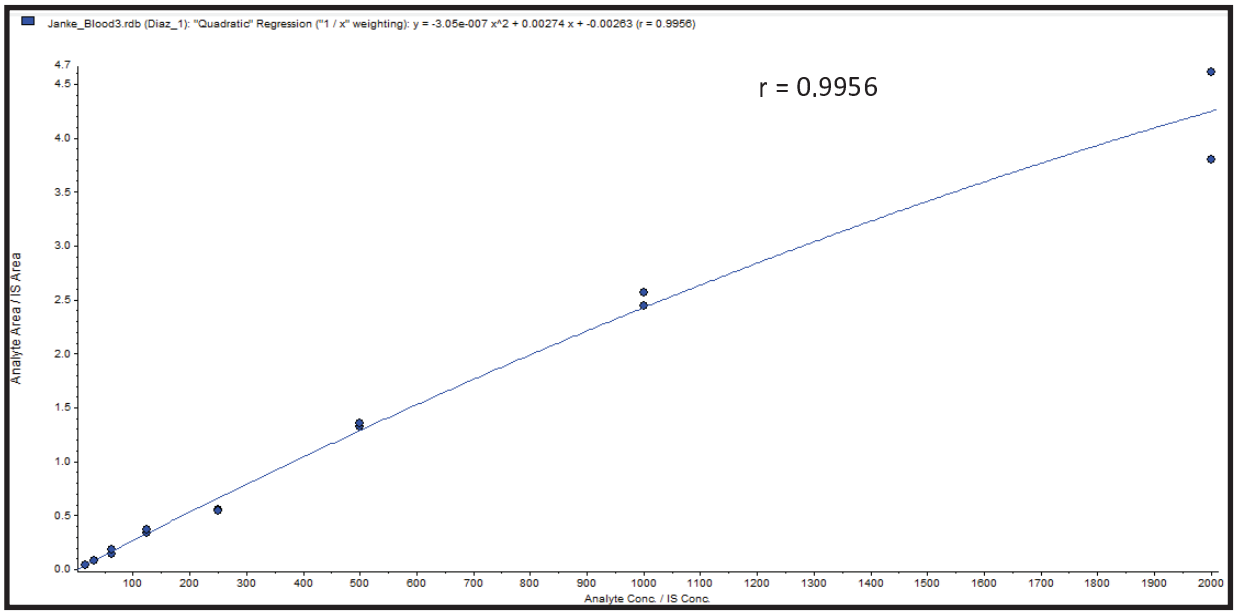


Figure A 18. Representative calibration curve for Diazepam: Validation 3, Day 3.

Table A 23.1. Diazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.7	1.18	7.1	107.2
31.3	S7	2 of 2	31	0.76	2.5	99.2
62.5	S6	2 of 2	61.1	12	19.7	97.8
125	S5	2 of 2	133	6.26	4.7	106.1
250	S4	2 of 2	207	1.57	0.8	82.9
500	S3	2 of 2	521	10.1	1.9	104.2
1000	S2	2 of 2	1040	40.3	3.9	103.9
2000	S1	2 of 2	1990	376	18.9	99.4

Table A 23.2. Summary of Diazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	17.1	1.69	9.9	109.4
45	L QC	6 of 6	38.9	5.46	14	86.5
800	M QC	6 of 6	776	39.5	5.1	97
1600	H QC	6 of 6	1750	252	14.4	109.7

Table A 24.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	16.6	1.76	10.7	106.5
31.3	S7	6 of 6	31.6	3.91	12.4	101.1
62.5	S6	6 of 6	61.6	5.58	8.9	98.6
125	S5	6 of 6	124	3.49	2.8	99.1
250	S4	6 of 6	224	12.9	5.9	89.6
500	S3	6 of 6	516	24.5	4.7	103.2
1000	S2	6 of 6	1030	89.4	8.7	102.9
2000	S1	6 of 6	1990	240	12.1	99.3

Table A 24.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	18 of 18	17.6	3.6	19.5	112.6
45	L QC	18 of 18	40.5	3.42	8.2	90.1
800	M QC	18 of 18	790	62.8	8	98.8
1600	H QC	18 of 18	1530	565	40.3	95.4

<u>Flunitrazepam</u>: Blood Validation 1, Day1

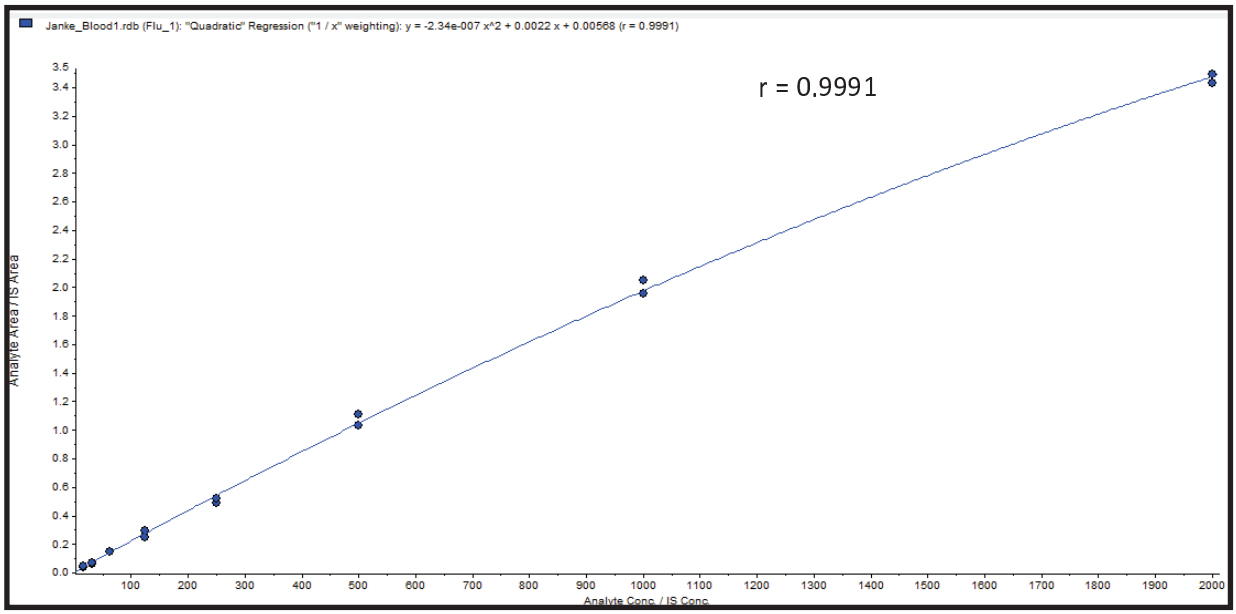


Figure A 19. Representative calibration curve for Flunitrazepam: Validation 1, Day 1.

Table A 25.1. Flunitrazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.7	2.63	15.8	107
31.3	S7	2 of 2	28.6	1.7	5.9	91.3
62.5	S6	2 of 2	66.7	0.02	0	106.8
125	S5	2 of 2	124	14.8	12	99
250	S4	2 of 2	231	10.2	4.4	92.3
500	S3	2 of 2	512	26.1	5.1	102.4
1000	S2	2 of 2	1020	36.8	3.6	101.7
2000	S1	2 of 2	1990	35	1.8	99.4

Table A 25.2. Summary of Flunitrazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	14.1	3.42	24.3	90.4
45	L QC	6 of 6	40.4	6.48	16	89.8
800	M QC	6 of 6	746	44.4	6	93.2
1600	H QC	6 of 6	1270	810	63.9	79.3

<u>Flunitrazepam</u>: Blood Validation 2, Day 2

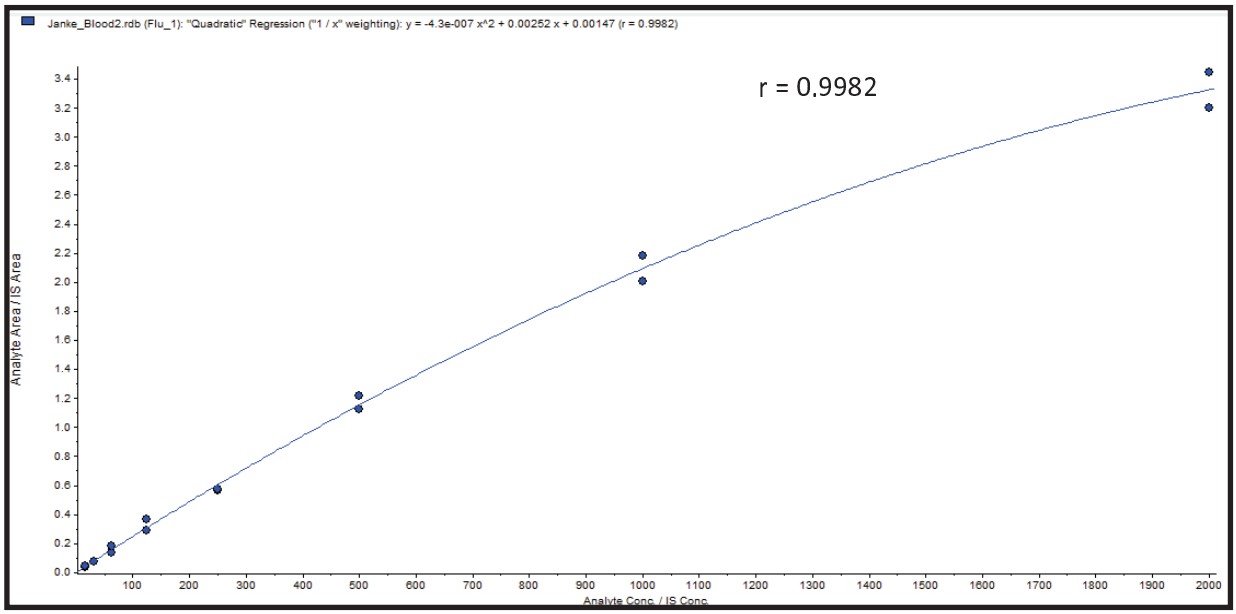


Figure A 20. Representative calibration curve for Flunitrazepam: Validation 2, Day 2.

Table A 26.1. Flunitrazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.7	2.47	15.7	100.8
31.3	S7	2 of 2	30.2	0.55	1.8	96.5
62.5	S6	2 of 2	63	12.4	19.6	100.8
125	S5	2 of 2	132	21.8	16.4	106
250	S4	2 of 2	235	3.53	1.5	94.2
500	S3	2 of 2	509	30.4	6	101.8
1000	S2	2 of 2	1000	74.3	7.4	100.1
2000	S1	2 of 2	2010	219	10.9	100.5

Table A 26.2. Summary of Flunitrazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	17.7	10.9	61.3	113.5
45	L QC	6 of 6	40.4	2.69	6.7	89.8
800	M QC	6 of 6	794	80	10.1	99.3
1600	H QC	6 of 6	1580	747	47.2	98.9

<u>Flunitrazepam</u>: Blood Validation 3, Day3

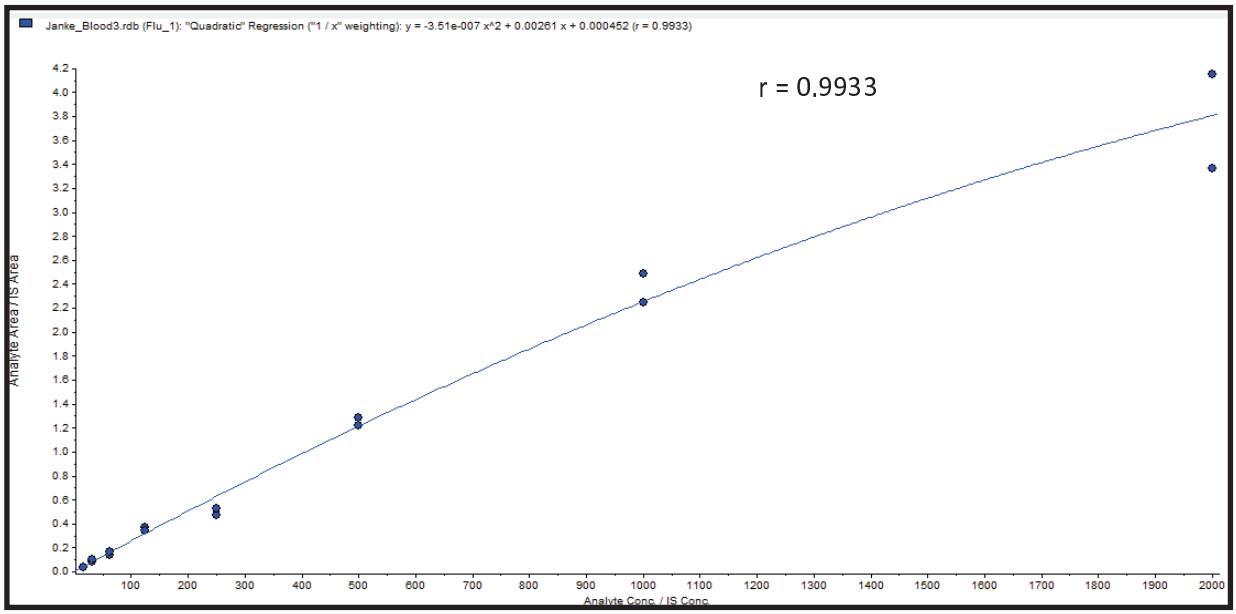


Figure A 21. Representative calibration curve for Flunitrazepam: Validation 3, Day 3.

Table A 27.1. Flunitrazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	14.7	0.9	6.1	94.1
31.3	S7	2 of 2	36.1	4.88	13.5	115.5
62.5	S6	2 of 2	58.4	7.5	12.8	93.4
125	S5	2 of 2	139	10	7.2	111.5
250	S4	2 of 2	196	15.8	8	78.6
500	S3	2 of 2	515	20.7	4	103.1
1000	S2	2 of 2	1060	91	8.6	106
2000	S1	2 of 2	1990	462	23.2	99.5

Table A 27.2. Summary of Flunitrazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	16.9	1.8	10.7	108.1
45	L QC	6 of 7	44.2	4.24	9.6	98.2
800	M QC	6 of 6	787	65.2	8.3	98.4
1600	H QC	6 of 6	1730	329	19	108.1

Table A 28.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	15.7	2	12.5	100.6
31.3	S7	6 of 6	31.6	2.37	7.1	101.1
62.5	S6	6 of 6	62.7	6.63	10.8	100.3
125	S5	6 of 6	132	15.5	11.9	105.5
250	S4	6 of 6	221	9.84	4.7	88.3
500	S3	6 of 6	512	25.7	5	102.4
1000	S2	6 of 6	1030	67.4	6.5	102.6
2000	S1	6 of 6	2000	239	12	99.8

Table A 28.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	18 of 18	16.2	5.36	32.1	104
45	L QC	18 of 18	41.7	4.47	10.8	92.6
800	M QC	18 of 18	776	63.2	8.1	97
1600	H QC	18 of 18	1530	629	43.4	95.4

Lorazepam: Blood Validation 1, Day1

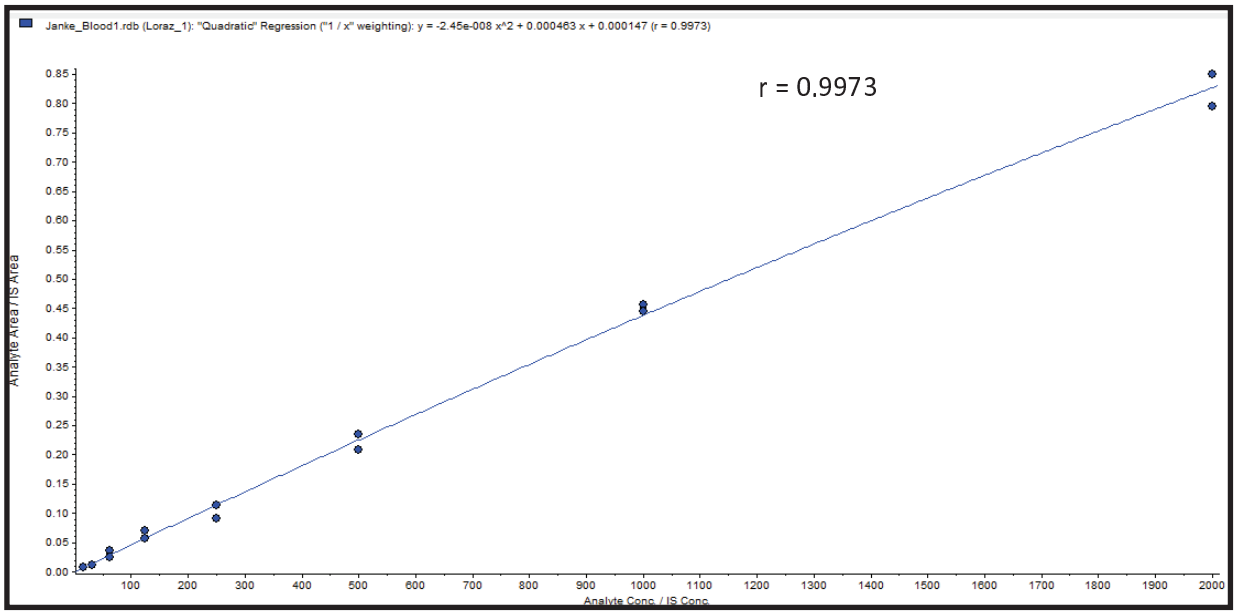


Figure A 22. Representative calibration curve for Lorazepam: Validation 1, Day 1.

Table A 29.1. Lorazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	17.4	1.48	8.5	111.5
31.3	S7	2 of 2	25.5	1.78	7	81.5
62.5	S6	2 of 2	65.3	16.2	24.8	104.5
125	S5	2 of 2	139	20.2	14.5	111.2
250	S4	2 of 2	225	36.1	16	90.1
500	S3	2 of 2	493	43.3	8.8	98.7
1000	S2	2 of 2	1030	18.3	1.8	103.2
2000	S1	2 of 2	1990	108	5.4	99.4

Table A 29.2. Summary of Lorazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	Accuracy
15.6	LLOQ	6 of 6	17.3	2.1	12.1	110.9
45	L QC	6 of 6	41.8	5.93	14.2	93
800	M QC	6 of 6	767	39.5	5.2	95.8
1600	H QC	6 of 6	1250	794	63.7	77.9

Lorazepam: Blood Validation 2, Day 2

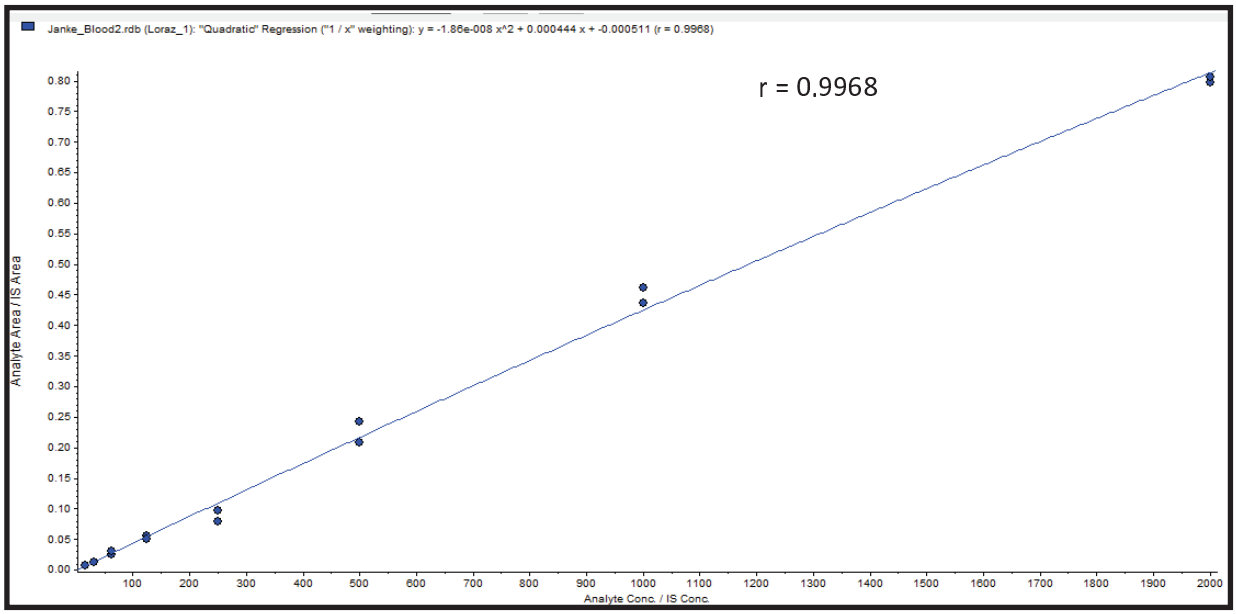


Figure A 23. Representative calibration curve for Lorazepam: Validation 2, Day 2.

Table A 30.1. Lorazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	17.7	0.52	2.9	113.5
31.3	S7	2 of 2	30.4	2.34	7.7	97
62.5	S6	2 of 2	64.7	9.39	14.5	103.5
125	S5	2 of 2	121	8.59	7.1	96.4
250	S4	2 of 2	203	29	14.3	81.1
500	S3	2 of 2	520	57.4	11	103.9
1000	S2	2 of 2	1060	43.1	4.1	106
2000	S1	2 of 2	1970	17.6	0.9	98.4

Table A 30.2. Summary of Lorazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	20.7	5.4	26	132.9
45	L QC	6 of 6	40.1	6.58	16.4	89.1
800	M QC	6 of 6	777	73.1	9.4	97.2
1600	H QC	6 of 6	1370	638	46.4	85.9

Lorazepam: Blood Validation 3, Day3

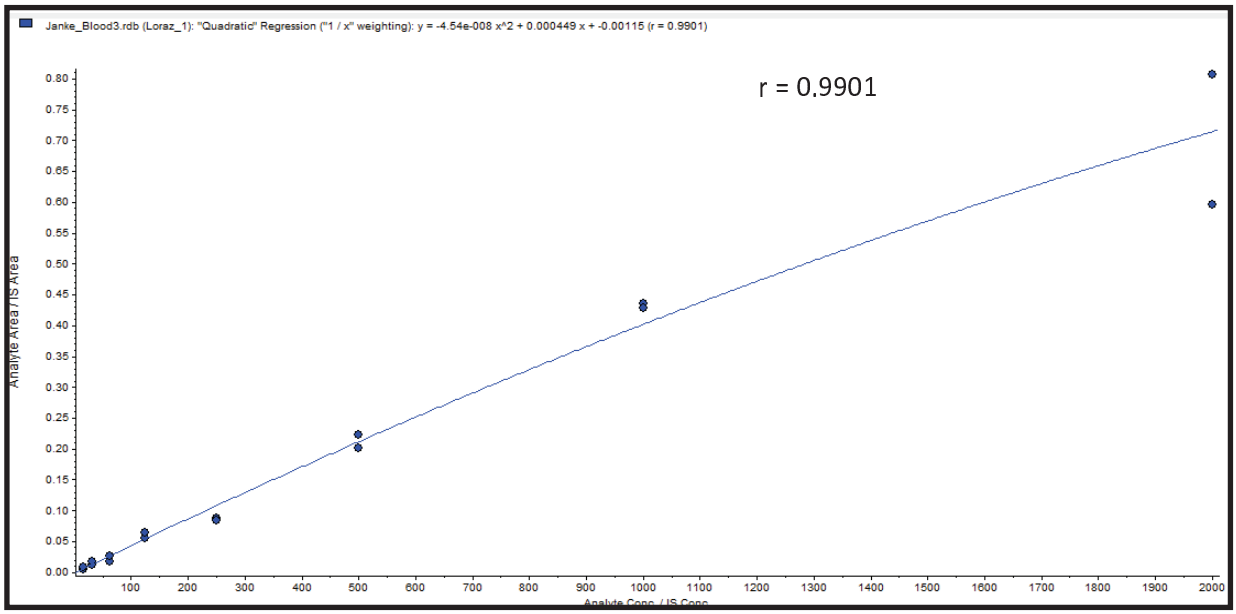


Figure A 24. Representative calibration curve for Lorazepam: Validation 3, Day 3.

Table A 31.1. Lorazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.6	5.13	30.9	106.4
31.3	S7	2 of 2	34.9	7.02	20.1	111.5
62.5	S6	2 of 2	53.2	13.9	26.1	85.2
125	S5	2 of 2	139	14.2	10.2	111.3
250	S4	2 of 2	198	4.56	2.3	79
500	S3	2 of 2	502	36.9	7.4	100.4
1000	S2	2 of 2	1090	14.3	1.3	108.6
2000	S1	2 of 2	1980	555	28.1	98.9

Table A 31.2. Summary of Lorazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	17.9	3.23	18.1	114.6
45	L QC	6 of 6	44.2	5.38	12.2	98.2
800	M QC	6 of 6	782	41.7	5.3	97.7
1600	H QC	6 of 6	1940	306	15.8	121.1

Table A 32.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	17.2	2.38	14.1	110.5
31.3	S7	6 of 6	30.3	3.71	11.6	96.7
62.5	S6	6 of 6	61.1	13.1	21.8	97.7
125	S5	6 of 6	133	14.3	10.6	106.3
250	S4	6 of 6	208	23.2	10.9	83.4
500	S3	6 of 6	505	45.9	9.1	101
1000	S2	6 of 6	1060	25.2	2.4	105.9
2000	S1	6 of 6	1980	227	11.5	98.9

Table A 32.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	7 of 7	18.6	3.58	18.7	119.5
45	L QC	6 of 6	42	5.97	14.3	93.4
800	M QC	6 of 6	775	51.4	6.6	96.9
1600	H QC	7 of 7	1520	579	42	95

Nitrazepam: Blood Validation 1, Day1

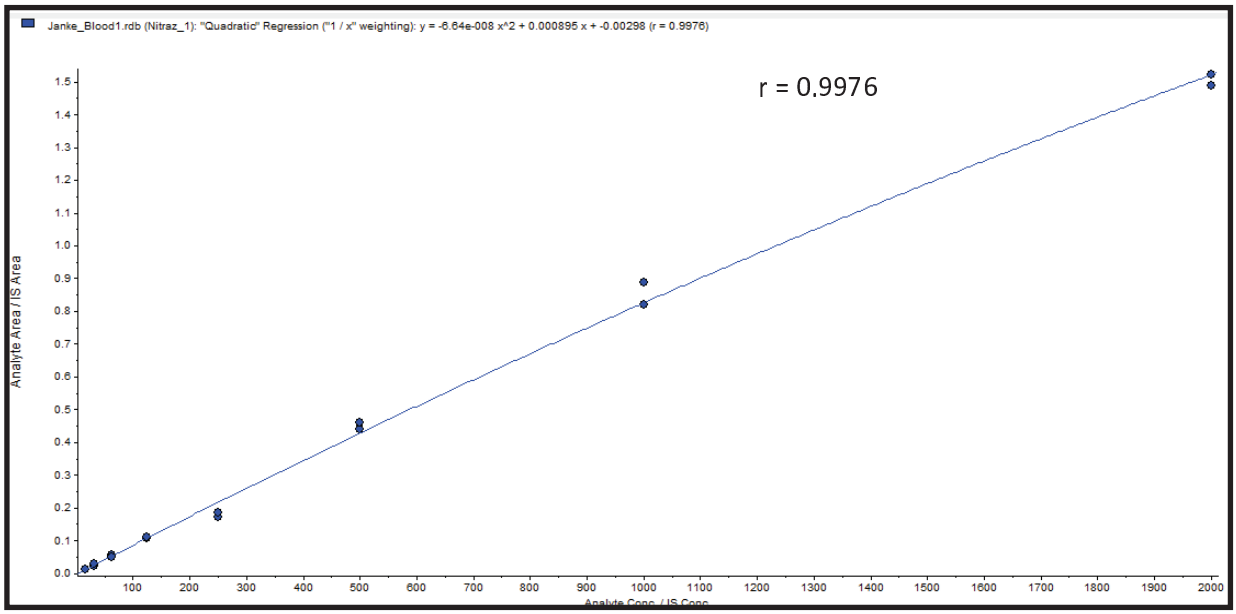


Figure A 25. Representative calibration curve for Nitrazepam: Validation 1, Day 1.

Table A 33.1. Nitrazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.7	0.06	0.3	107.1
31.3	S7	2 of 2	31.2	5.48	17.6	99.5
62.5	S6	2 of 2	63.9	6.54	10.2	102.2
125	S5	2 of 2	127	2.72	2.1	101.3
250	S4	2 of 2	204	11	5.4	81.8
500	S3	2 of 2	526	18	3.4	105.3
1000	S2	2 of 2	1040	65.1	6.3	103.9
2000	S1	2 of 2	1980	37.7	1.9	98.8

Table A 33.2. Summary of Nitrazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	17.3	1.81	10.5	110.7
45	L QC	6 of 6	42.4	3.75	8.8	94.1
800	M QC	6 of 6	739	21.9	3	92.3
1600	H QC	6 of 6	1280	818	64.2	79.7

Nitrazepam: Blood Validation 2, Day 2

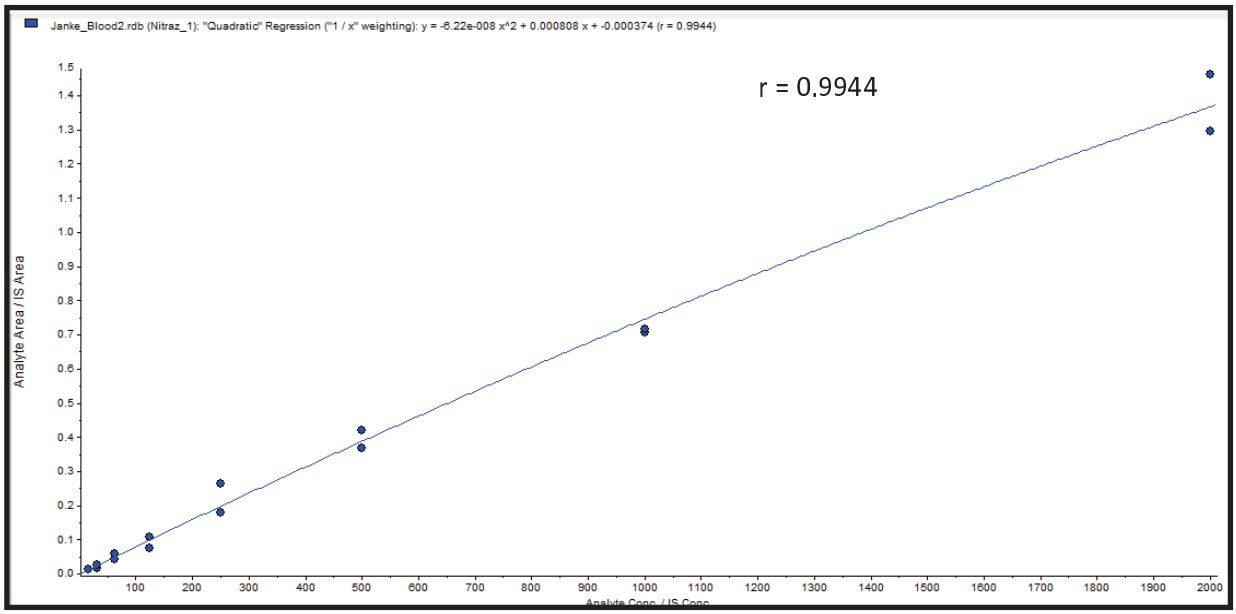


Figure A 26. Representative calibration curve for Nitrazepam: Validation 2, Day 2.

Table A 34.1. Nitrazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.8	0.24	1.4	107.7
31.3	S7	2 of 2	27.7	6.51	23.5	88.6
62.5	S6	2 of 2	63.5	15.6	24.5	101.6
125	S5	2 of 2	115	30	26.1	92.1
250	S4	2 of 2	281	78.3	27.9	112.3
500	S3	2 of 2	508	48.4	9.5	101.6
1000	S2	2 of 2	952	9.84	1	95.2
2000	S1	2 of 2	2030	209	10.3	101.3

Table A 34.2. Summary of Nitrazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	19.9	11.6	58.1	127.8
45	L QC	6 of 6	42	3.87	9.2	93.4
800	M QC	6 of 6	894	40.6	4.5	111.7
1600	H QC	6 of 6	1470	682	46.3	92.2

Nitrazepam: Blood Validation 3, Day3

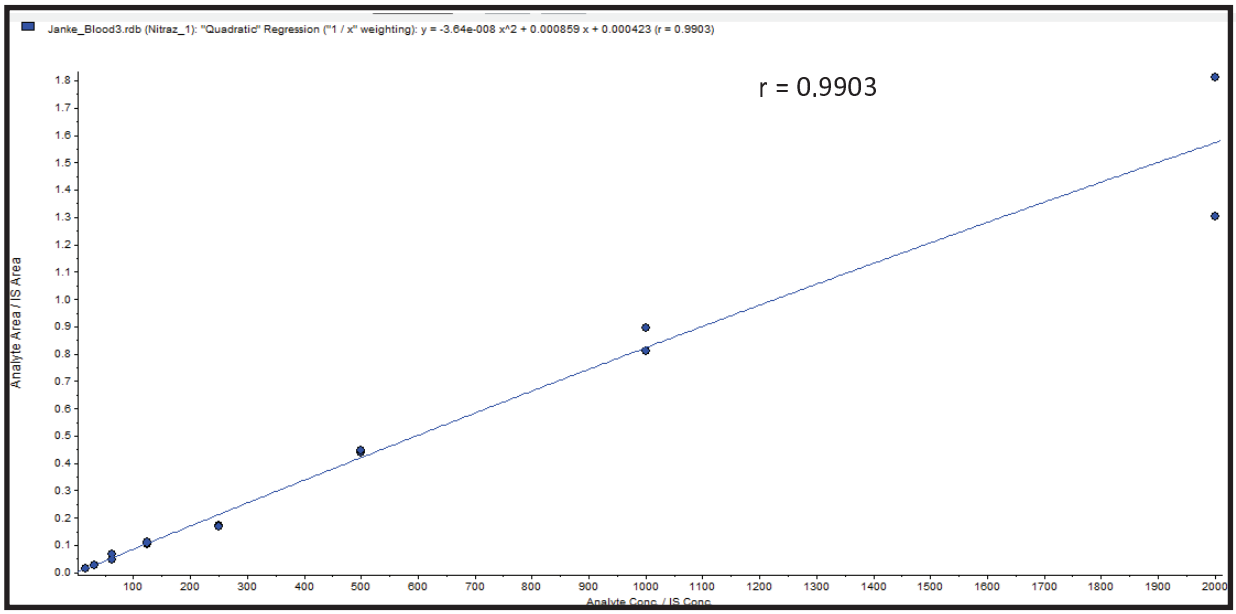


Figure A 27. Representative calibration curve for Nitrazepam: Validation 3, Day 3.

Table A 35.1. Nitrazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.7	0.19	1.1	107.1
31.3	S7	2 of 2	30.6	2.01	6.6	97.9
62.5	S6	2 of 2	66.3	14.1	21.3	106.1
125	S5	2 of 2	125	5.04	4	99.8
250	S4	2 of 2	202	3.13	1.6	80.7
500	S3	2 of 2	529	6.4	1.2	105.7
1000	S2	2 of 2	1040	76.8	7.4	103.8
2000	S1	2 of 2	1980	504	25.4	99.2

Table A 35.2. Summary of Nitrazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	14.7	1.64	11.1	94.5
45	L QC	6 of 6	44.4	7.17	16.2	98.6
800	M QC	6 of 6	810	33.9	4.2	101.2
1600	H QC	6 of 6	1830	198	10.8	114.2

Table A36.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	16.7	0.16	1	107.3
31.3	S7	6 of 6	29.8	4.67	15.9	95.3
62.5	S6	6 of 6	64.6	12.1	18.7	103.3
125	S5	6 of 6	122	12.6	10.7	97.7
250	S4	6 of 6	229	30.8	11.6	91.6
500	S3	6 of 6	521	24.3	4.7	104.2
1000	S2	6 of 6	1010	50.6	4.9	100.9
2000	S1	6 of 6	2000	250	12.5	99.8

Table A 36.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	18 of 18	17.3	5.01	26.6	111
45	L QC	18 of 18	42.9	4.93	11.4	95.4
800	M QC	18 of 18	814	32.1	3.9	101.8
1600	H QC	18 of 18	1530	566	40.4	95.3

Oxazepam: Blood Validation 1, Day1

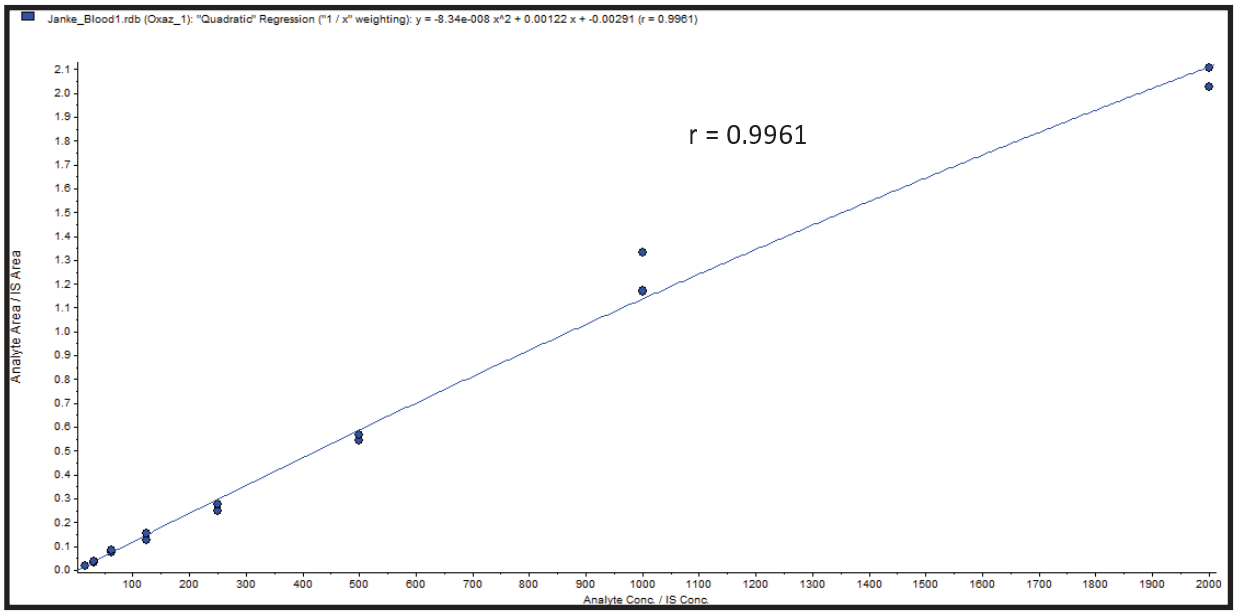


Figure A 28. Representative calibration curve for Oxazepam: Validation 1, Day 1.

Table A 37.1. Oxazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.8	0.05	0.3	107.5
31.3	S7	2 of 2	31.2	4.2	13.5	99.6
62.5	S6	2 of 2	67.5	5.16	7.6	108
125	S5	2 of 2	118	14.8	12.5	94.7
250	S4	2 of 2	219	17.3	7.9	87.4
500	S3	2 of 2	472	15.9	3.4	94.4
1000	S2	2 of 2	1110	110	9.9	110.9
2000	S1	2 of 2	1950	63	3.2	97.6

Table A 37.2. Summary of Oxazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	73	149	203.6	468.1
45	L QC	6 of 6	39	4.22	10.8	86.6
800	M QC	6 of 6	774	70.2	9.1	96.8
1600	H QC	6 of 6	1340	864	64.3	84

<u>Oxazepam</u>: Blood Validation 2, Day 2

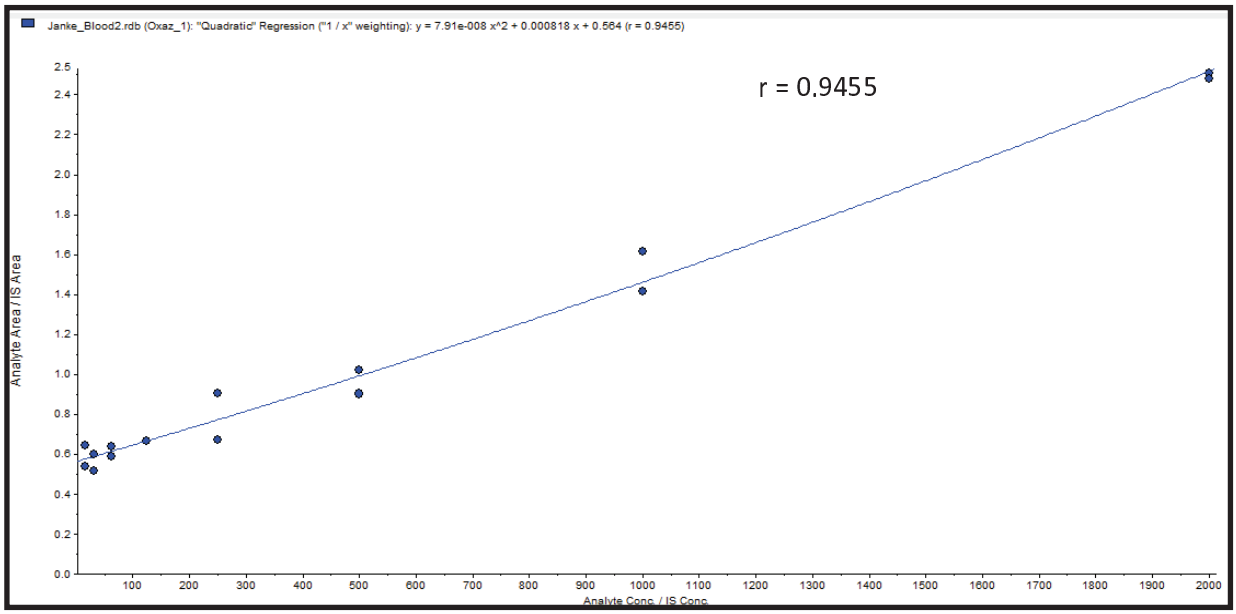


Figure A 29. Representative calibration curve for Oxzepam: Validation 2, Day 2.

Table A 38.1. Oxazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	1 of 2	96.1	N/A	N/A	615.9
31.3	S7	1 of 2	46.7	N/A	N/A	149.3
62.5	S6	2 of 2	59	42.7	72.4	94.4
125	S5	2 of 2	125	1.01	0.8	99.8
250	S4	2 of 2	265	189	71.5	105.9
500	S3	2 of 2	466	96	20.6	93.3
1000	S2	2 of 2	1060	144	13.6	105.6
2000	S1	2 of 2	1980	17.9	0.9	99

Table A 38.2. Summary of Oxazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 6	30.2	0.55	1.8	193.5
45	L QC	2 of 6	17.1	16.5	96.5	38
800	M QC	6 of 6	869	70	8.1	108.6
1600	H QC	6 of 6	1330	588	44.1	83.3

<u>Oxazepam</u>: Blood Validation 3, Day3

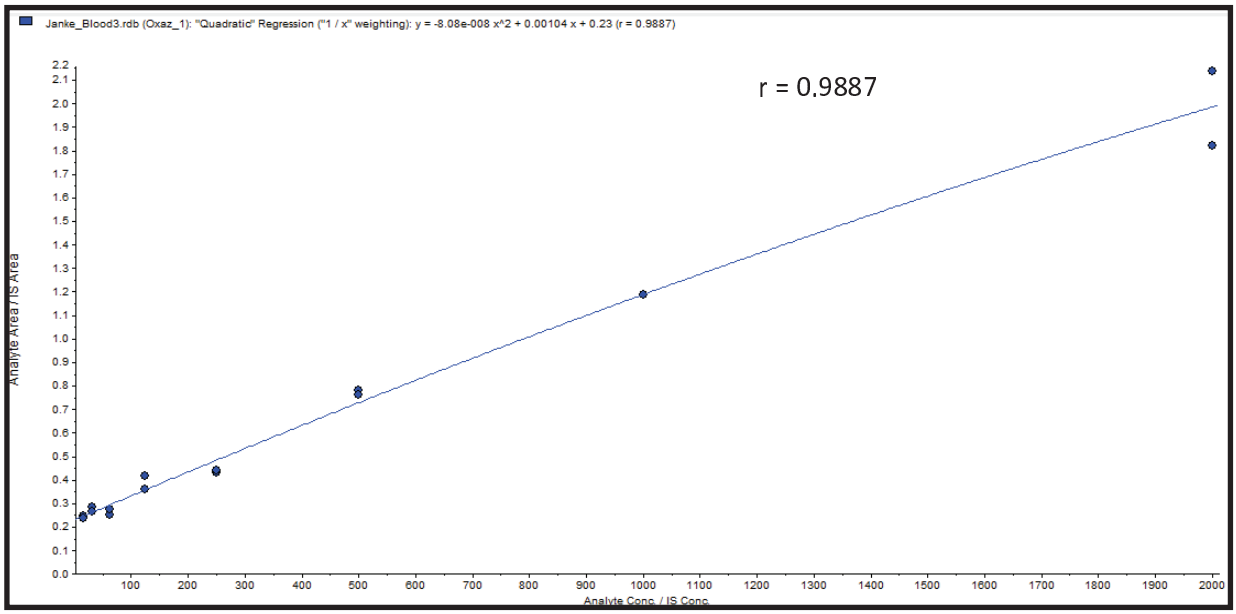


Figure A 30. Representative calibration curve for Oxazepam: Validation 3, Day 3.

Table A 39.1. Oxazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	14.5	6.55	45.1	93.2
31.3	S7	2 of 2	44.8	13.2	29.6	143.1
62.5	S6	2 of 2	31.7	16.1	50.7	50.8
125	S5	2 of 2	155	40.8	26.4	123.7
250	S4	2 of 2	203	7.91	3.9	81
500	S3	2 of 2	544	14.2	2.6	108.8
1000	S2	2 of 2	1000	2.36	0.2	100
2000	S1	2 of 2	2000	309	15.5	99.9

Table A 39.2. Summary of Oxazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 6	20.8	7.78	37.3	133.6
45	L QC	6 of 6	43.5	20.6	47.5	96.6
800	M QC	6 of 6	839	79.4	9.5	104.8
1600	H QC	6 of 6	1980	308	15.6	123.5

Table A 40.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	5 of 6	42.5	3.3	22.7	272.2
31.3	S7	5 of 6	40.9	8.72	21.5	130.7
62.5	S6	6 of 6	52.7	21.3	43.6	84.4
125	S5	6 of 6	133	18.9	13.2	106
250	S4	6 of 6	229	71.5	27.8	91.4
500	S3	6 of 6	494	42	8.9	98.8
1000	S2	6 of 6	1060	85.3	7.9	105.5
2000	S1	6 of 6	1980	130	6.5	98.8

Table A 40.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	10 of 18	41.3	52.3	80.9	265.1
45	L QC	14 of 18	33.2	13.8	51.6	73.7
800	M QC	18 of 18	827	73.2	8.9	103.4
1600	H QC	18 of 18	1550	587	41.3	97

<u>Temazepam</u>: Blood Validation 1, Day1

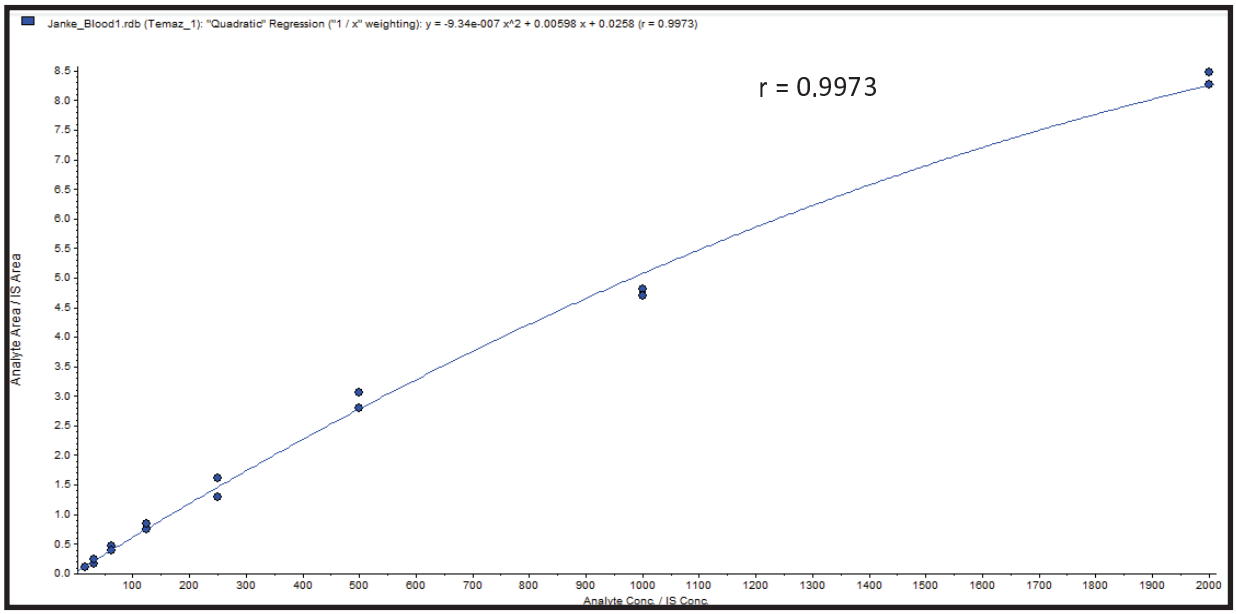


Figure A 31. Representative calibration curve for Temazepam: Validation 1, Day 1.

Table A 41.1. Temazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.8	0.03	0.2	88.8
31.3	S7	2 of 2	30.4	8.36	27.5	97.2
62.5	S6	2 of 2	67.4	8.66	12.8	107.9
125	S5	2 of 2	133	11.2	8.4	106.2
250	S4	2 of 2	249	41.1	16.5	99.5
500	S3	2 of 2	530	35.6	6.7	105.9
1000	S2	2 of 2	924	19.9	2.2	92.4
2000	S1	2 of 2	2060	69	3.4	102.8

Table A 41.2. Summary of Temazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	Accuracy
15.6	LLOQ	6 of 6	11	2.23	20.2	70.7
45	L QC	6 of 6	36.7	6.63	18.1	81.5
800	M QC	6 of 6	747	37.6	5	93.3
1600	H QC	6 of 6	1260	812	64.4	78.8

Temazepam: Blood Validation 2, Day 2

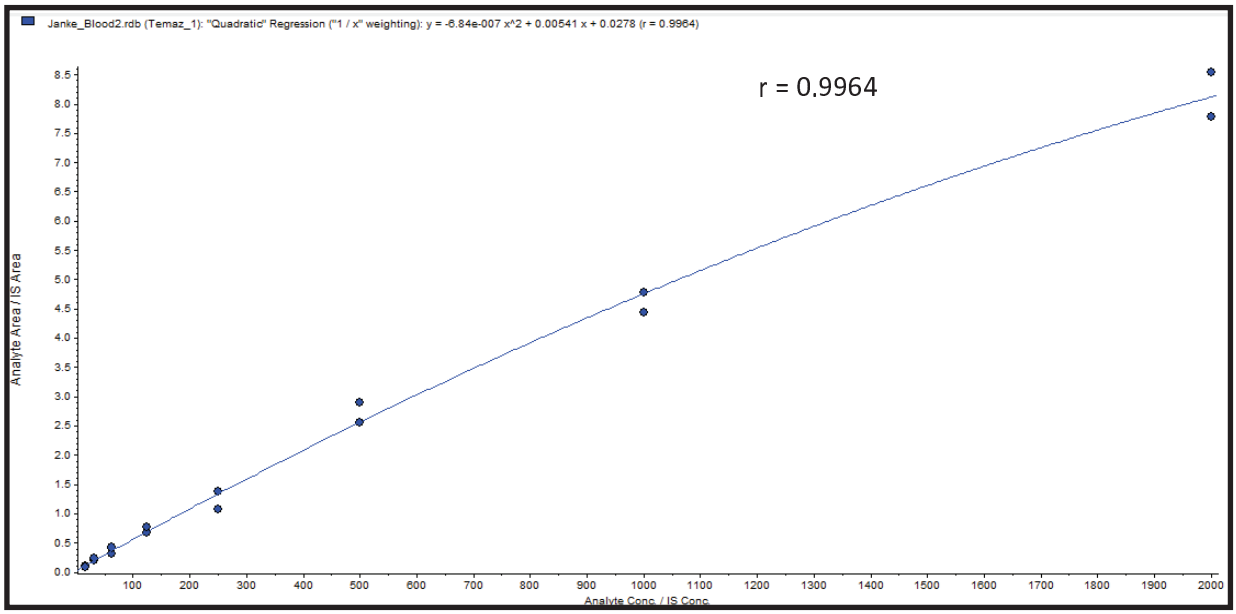


Figure A 32. Representative calibration curve for Temazepam: Validation 2, Day 2.

Table A 42.1. Temazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.1	3.1	23.6	84
31.3	S7	2 of 2	35.2	5.64	16	112.4
62.5	S6	2 of 2	64.4	12.8	19.9	103
125	S5	2 of 2	131	14.8	11.3	105.1
250	S4	2 of 2	228	41	17.9	91.4
500	S3	2 of 2	535	52.9	9.9	106.9
1000	S2	2 of 2	964	60.1	6.2	96.4
2000	S1	2 of 2	2020	202	10	101.1

Table A 42.2. Summary of Temazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	16	9.61	59.9	102.8
45	L QC	6 of 6	42	3.92	9.3	93.3
800	M QC	6 of 6	783	64.6	8.3	97.9
1600	H QC	6 of 6	1430	666	46.5	89.6

<u>Temazepam</u>: Blood Validation 3, Day3

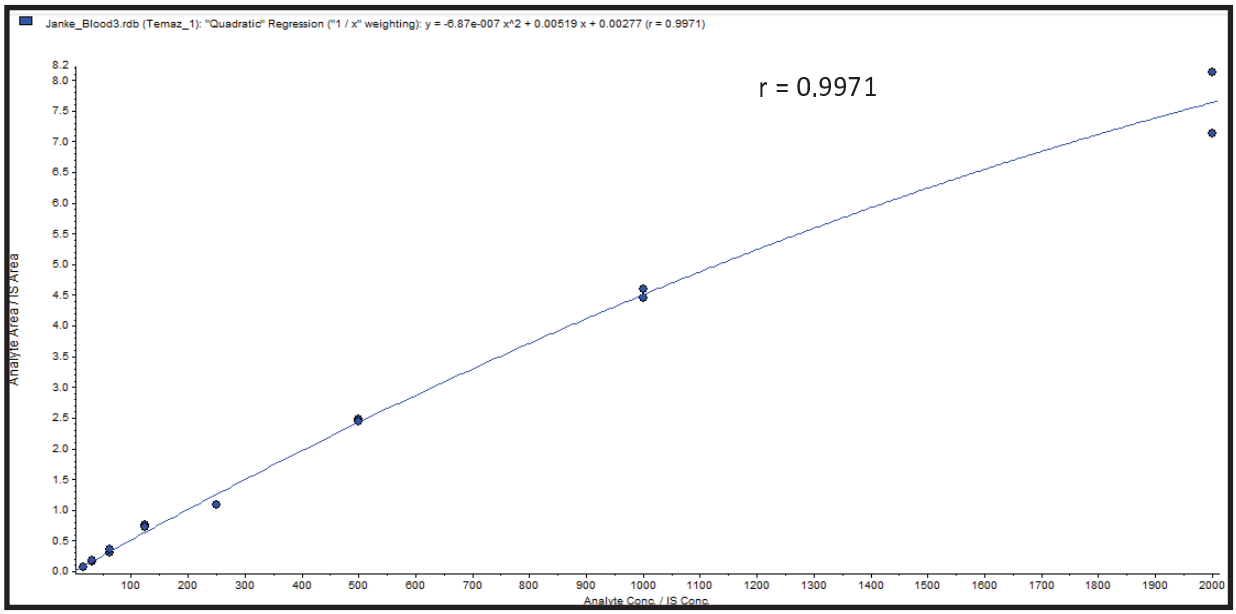


Figure A 33. Representative calibration curve for Temazepam: Validation 3, Day 3.

Table A 43.1. Temazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.8	1.21	8.8	88.8
31.3	S7	2 of 2	31.9	3.67	11.5	102
62.5	S6	2 of 2	65.1	7.49	11.5	104.1
125	S5	2 of 2	146	3.8	2.6	116.6
250	S4	2 of 2	216	0.8	0.4	86.3
500	S3	2 of 2	509	4.16	0.8	101.7
1000	S2	2 of 2	1010	26.1	2.6	100.7
2000	S1	2 of 2	2010	292	14.6	100.4

Table A 43.2. Summary of Temazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	16.5	2.98	18.1	105.7
45	L QC	6 of 6	46.9	6.99	14.9	104.3
800	M QC	6 of 6	795	41	5.2	99.3
1600	H QC	6 of 6	1840	302	16.4	115

Table A 44.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	13.6	1.45	10.9	87.2
31.3	S7	6 of 6	32.5	5.89	18.3	103.9
62.5	S6	6 of 6	65.6	9.65	14.7	105
125	S5	6 of 6	137	9.94	7.4	109.3
250	S4	6 of 6	231	27.6	11.6	92.4
500	S3	6 of 6	524	30.9	5.8	104.9
1000	S2	6 of 6	965	35.4	3.7	96.5
2000	S1	6 of 6	2030	188	9.3	101.4

Table A 44.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	18 of 18	14.5	4.94	32.7	93.1
45	L QC	18 of 18	41.9	5.84	14.1	93
800	M QC	18 of 18	775	47.7	6.1	96.8
1600	H QC	18 of 18	1510	593	42.4	94.5

Here follows a list of %Accuracy values that did not fall within the acceptance criteria values for calibration standards and quality controls:

Table A 45%Accuracy values that did not fit acceptance criteria and an indication of the amount of percent with which it was too high or too low to get to either the lowest or highest accepted percentage.

Analyte	Validation Batch	STD/QC Fail	%Accuracy	+/- % out*
Nortriptyline	Validation 1	L QC	83.50951	+ 1
	Validation 3	S5	117.6253	- 3
		S4	70.30619	+ 15
		LLOQ	122.2342	-2
	Overall	S4	82.33573	+ 3
Amitriptyline	Validation 1	H QC	81.33081	+ 4
	Validation 3	S4	69.01028	+ 15
	Overall	S4	81.26692	+ 4
Citalopram	Validation 1	S8	123.3066	- 8
		S4	84.08859	+ 1
		LLOQ	132.9086	-13
		H QC	77.28092	+ 8
	Validation 3	S8	127.988	- 12
		S4	52.8906	+ 32
		S2	118.0322	-3
		LLOQ	134.1046	- 14
		H QC	140.9582	- 26
	Overall	S8	122.0331	- 7
		S4	75.45518	+ 10
		LLOQ	124.5652	-15
Alprazolam	Validation 1	S7	83.47535	+ 2
		H QC	75.33067	+ 10
	Validation 3	S8	78.24093	+ 7
		S5	126.068	-11
		S4	79.59255	+ 5
Clonazepam	Validation 1	S8	118.0873	- 3
		H QC	79.78537	+ 5
	Validation 2	S4	81.46645	+ 4
	Validation 3	S4	82.80749	+ 2
Diazepam	Validation 1	H QC	79.79211	+ 5
	Validation 2	LLOQ	125.3529	- 5
	Validation 3	S4	82.8853	+ 2
Flunitrazepam	Validation 1	H QC	79.31646	+ 6
	Validation 3	S4	78.57018	+ 6
Lorazepam	Validation 1	S7	81.47415	+ 4
		H QC	77.94116	+ 7
	Validation 2	S4	81.09102	+ 4
		LLOQ	132.9152	-13
	Validation 3	S4	79.02308	+ 6
		H QC	121.0761	-6
	Overall	S4	83.39728	+ 2

Nitrazepam	Validation 1	S4 H QC	81.78931 79.69189	+ 3 + 5
	Validation 2	LLOQ	127.8416	- 8
	Validation 3	S4	80.66084	+ 4
Oxazepam	Validation 1	LLOQ H QC	468.1168 84.02877	- 348 + 1
	Validation 2	S8 S7 LLOQ L QC H QC	615.8908 149.2953 193.48 37.95139 83.31057	- 501 - 6 - 78 + 47 + 2
	Validation 3	S7 S6 S5 S4 LLOQ H QC	143.1146 50.79811 123.6774 81.0089 133.5946 123.5258	- 28 + 34 -9 + 4 - 14 - 9
	Overall	S8 S7 S6 LLOQ L QC	272.1747 130.6643 84.3933 265.0638 73.71277	- 157 - 16 + 1 -150 + 11
Temazepam	Validation 1	LLOQ L QC H QC	70.69303 81.53047 78.83624	+ 14 + 3 + 6
	Validation 2	S8	84.04111	+ 1

*Rounding up to get to lowest or highest accepted %Accuracy values

B: Serum Accuracy and Precision Validation Results

Here follows a list for the series of results that is presented for the Serum Accuracy and Precision Results. It can be seen that the following sequence of results is presented for each analyte:

- A representative calibration curve (including r^2 values)
- Calibration standard accuracy and precision results
- Summary of intra-validation quality control standards

} Presented for validation 1, 2 and 3 for each analyte

- Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3
- Overall Quality Control Accuracy and Precision Estimation

Nortriptyline: Serum Validation 1, Day1

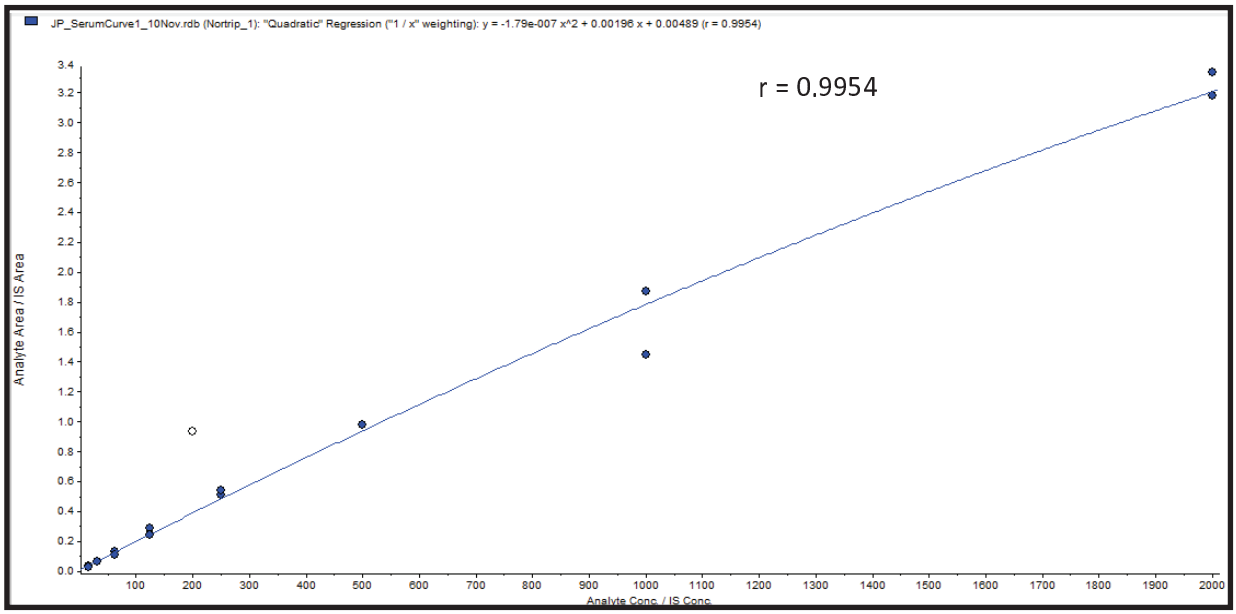


Figure B 1. Representative calibration curve for Nortriptyline: Validation 1, Day 1.

Table B 1.1. Nortriptyline Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.7	3.36	24.5	88.1
31.3	S7	2 of 2	31.8	0.12	0.4	101.6
62.5	S6	2 of 2	60.7	7.94	13.1	97.2
125	S5	2 of 2	135	18.7	13.9	107.8
250	S4	2 of 2	272	11.6	4.3	108.6
500	S3	1 of 1	524	N/A	N/A	104.8
1000	S2	2 of 2	924	183	19.8	92.4
2000	S1	2 of 2	2040	91.9	4.5	102.1

Table B 1.2. Summary of Nortriptyline intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	14.8	0.46	3.1	94.9
45	L QC	2 of 2	47.9	8.42	17.6	106.5
800	M QC	2 of 2	913	151	16.6	114.1
1600	H QC	2 of 2	2110	207	9.8	131.9

Nortriptyline: Serum Validation 2, Day 2

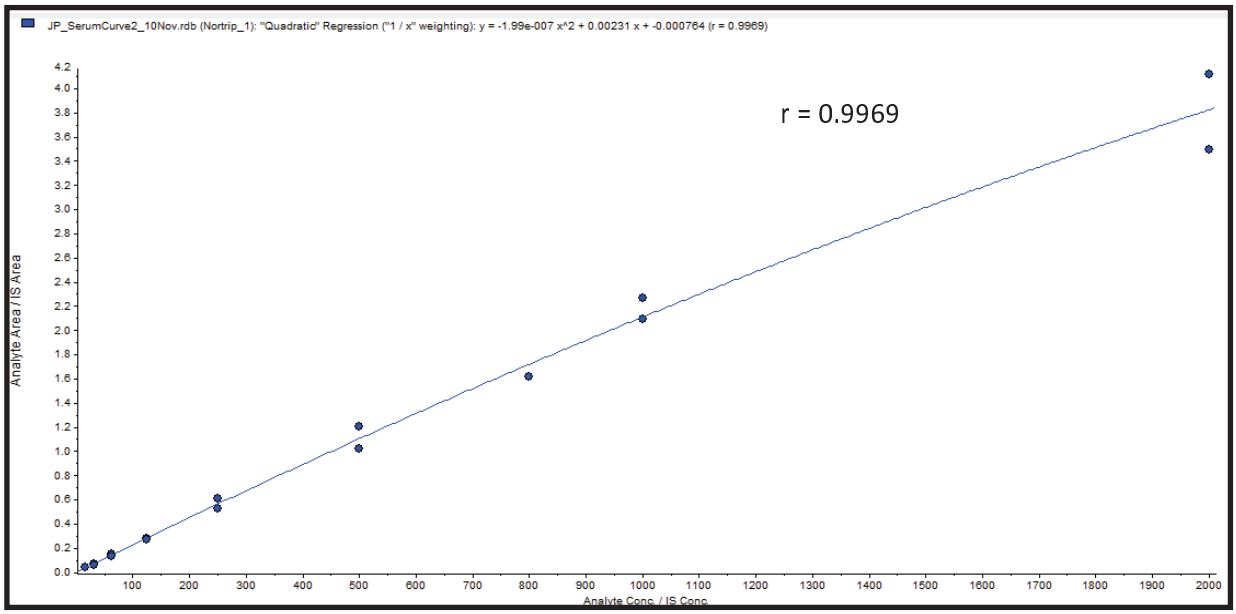


Figure B 2. Representative calibration curve for Nortriptyline: Validation 2, Day 2.

Table B 2.1. Nortriptyline Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.9	2.2	13	108.2
31.3	S7	2 of 2	30.4	2.59	8.5	97.3
62.5	S6	2 of 2	61.9	6.37	10.3	99.1
125	S5	2 of 2	119	0.66	0.6	95.5
250	S4	2 of 2	247	25.1	10.2	98.8
500	S3	2 of 2	497	61.4	12.4	99.4
1000	S2	2 of 2	1020	63.2	6.2	102.5
2000	S1	2 of 2	1990	296	14.9	99.6

Table B 2.2. Summary of Nortriptyline intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	16	0.21	1.3	102.8
45	L QC	2 of 2	45.3	1.47	3.3	100.7
800	M QC	2 of 2	782	11.5	1.5	97.8
1600	H QC	2 of 2	1540	133	8.6	96.1

Nortriptyline: Serum Validation 3, Day 2

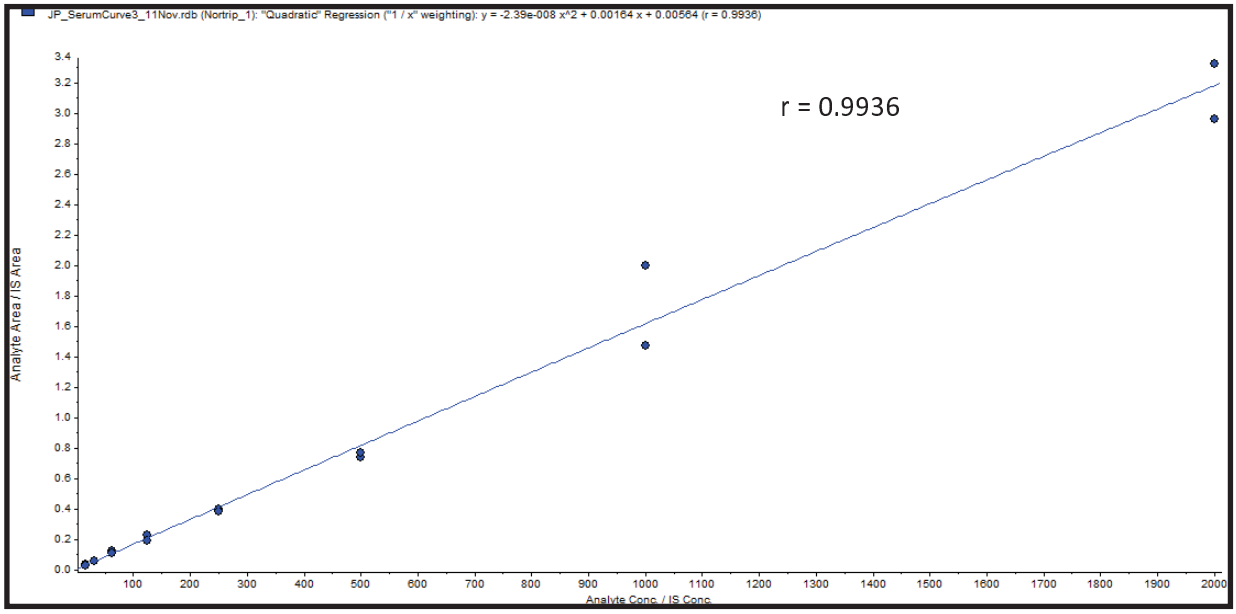


Figure B 3. Representative calibration curve for Nortriptyline: Validation 3, Day 2.

Table B 3.1. Nortriptyline Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.6	2.88	18.5	100
31.3	S7	2 of 2	30.6	0.69	2.3	97.9
62.5	S6	2 of 2	68.1	5.19	7.6	108.9
125	S5	2 of 2	125	14.4	11.5	100
250	S4	2 of 2	237	8.25	3.5	94.9
500	S3	2 of 2	460	13.5	2.9	92.1
1000	S2	2 of 2	1070	235	21.9	107.4
2000	S1	2 of 2	1970	168	8.5	98.7

Table B 3.2. Summary of Nortriptyline intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	15.7	0.25	1.6	100.4
45	L QC	2 of 2	48	10.8	22.4	106.8
800	M QC	2 of 2	803	1.33	0.2	100.4
1600	H QC	2 of 2	1780	73.2	4.1	111.5

Table B 4.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	15.4	2.81	18.7	98.8
31.3	S7	6 of 6	31	1.13	3.7	98.9
62.5	S6	6 of 6	63.6	6.5	10.3	101.7
125	S5	6 of 6	126	11.2	8.6	101.1
250	S4	6 of 6	410	28.9	6.5	100
500	S3	5 of 5	874	149	14	104.9
1000	S2	6 of 6	1630	216	14.4	96.9
2000	S1	6 of 6	2040	91.9	4.5	102.1

Table B 4.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	15.5	0.31	2	99.4
45	L QC	6 of 6	47.1	6.88	14.4	104.6
800	M QC	6 of 6	833	54.6	6.1	104.1
1600	H QC	6 of 6	1810	138	7.5	113.2

Amitriptyline: Serum Validation 1, Day1

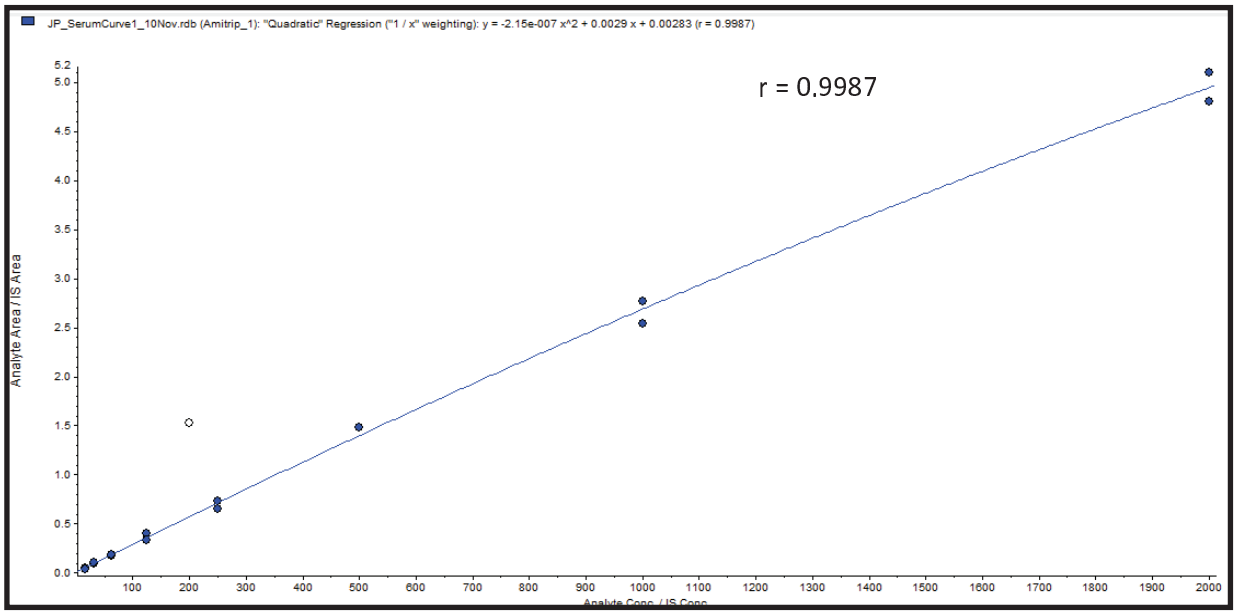


Figure B 4. Representative calibration curve for Amitriptyline: Validation 1, Day 1.

Table B 5.1. Amitriptyline Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	14.9	4.5	30.2	95.5
31.3	S7	2 of 2	33.3	2.73	8.2	106.3
62.5	S6	2 of 2	61.5	3.13	5.1	98.3
125	S5	2 of 2	126	17.3	13.7	101
250	S4	2 of 2	242	21.4	8.8	96.9
500	S3	1 of 1	530	N/A	N/A	106.1
1000	S2	2 of 2	987	64.2	6.5	98.7
2000	S1	2 of 2	2000	103	5.1	100.2

Table B 5.2. Summary of Amitriptyline intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	16.4	3.79	23.1	105
45	L QC	2 of 2	45.9	3.72	8.1	102
800	M QC	2 of 2	909	94	10.3	113.6
1600	H QC	2 of 2	2050	211	10.3	128.1

Amitriptyline: Serum Validation 2, Day 2

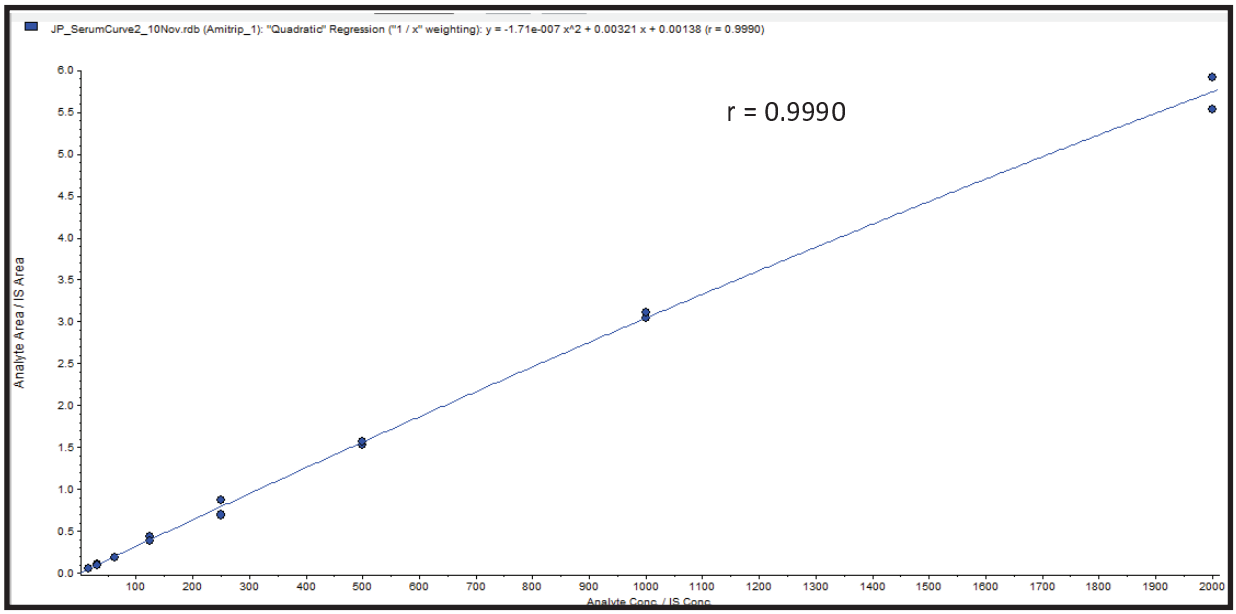


Figure B 5. Representative calibration curve for Amitriptyline: Validation 2, Day 2.

Table B 6.1. Amitriptyline Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.8	0.24	1.4	107.9
31.3	S7	2 of 2	30.2	2.22	7.4	96.5
62.5	S6	2 of 2	59.2	1.16	2	94.7
125	S5	2 of 2	127	12.3	9.6	102
250	S4	2 of 2	247	39.7	16.1	98.6
500	S3	2 of 2	496	9.84	2	99.3
1000	S2	2 of 2	1010	16.1	1.6	101.4
2000	S1	2 of 2	1990	106	5.3	99.7

Table B 6.2. Summary of Amitriptyline intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	15.2	1.22	8	97.5
45	L QC	2 of 2	45.4	3.03	6.7	100.9
800	M QC	2 of 2	841	47.2	5.6	105.1
1600	H QC	2 of 2	1600	188	11.7	100

<u>Amitriptyline</u>: Serum Validation 3, Day 2

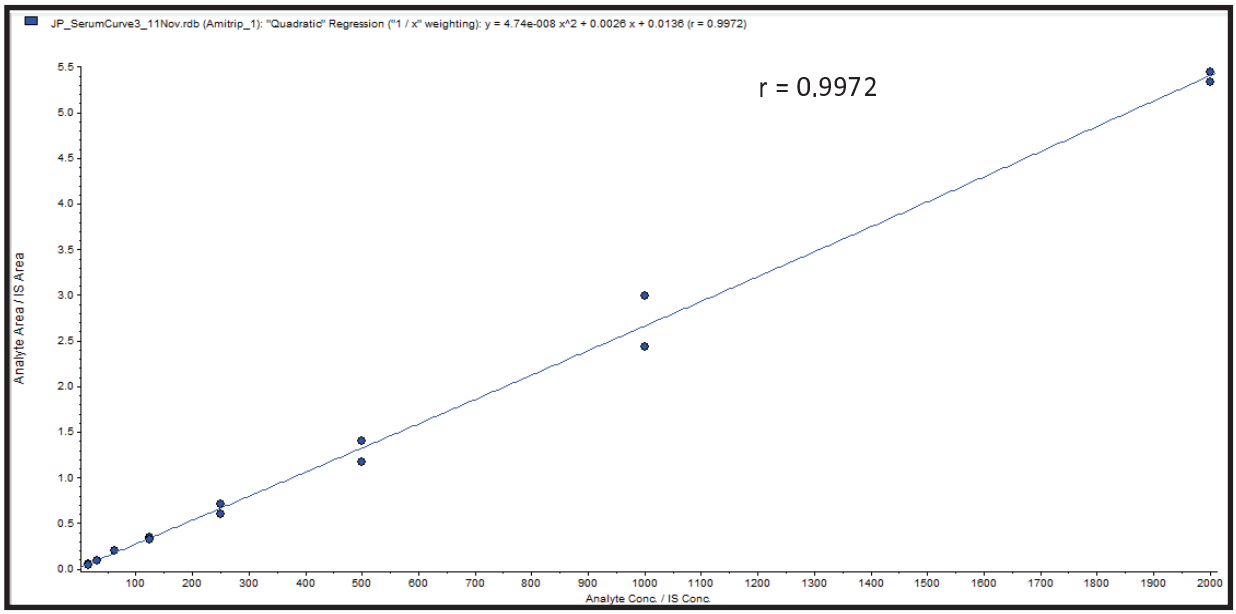


Figure B 6. Representative calibration curve for Amitriptyline: Validation 3, Day 2.

Table B 7.1. Amitriptyline Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	14.5	4.67	32.3	92.8
31.3	S7	2 of 2	29.9	0.18	0.6	95.4
62.5	S6	2 of 2	72.4	0.53	0.7	115.9
125	S5	2 of 2	124	4.03	3.3	98.9
250	S4	2 of 2	246	27.4	11.1	98.4
500	S3	2 of 2	484	61.9	12.8	96.8
1000	S2	2 of 2	1020	146	14.3	101.9
2000	S1	2 of 2	1990	27.1	1.4	99.7

Table B 7.2. Summary of Amitriptyline intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	15.7	0.25	1.6	100.4
45	L QC	2 of 2	48	10.8	22.4	106.8
800	M QC	2 of 2	803	1.33	0.2	100.4
1600	H QC	2 of 2	1780	73.2	4.1	111.5

Table B 8.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	15.4	3.14	21.3	98.7
31.3	S7	6 of 6	31.1	1.71	5.4	99.4
62.5	S6	6 of 6	64.3	1.61	2.6	103
125	S5	6 of 6	126	11.2	8.9	100.6
250	S4	6 of 6	246	33.6	13.6	98.5
500	S3	5 of 5	408	31	7.9	97.7
1000	S2	6 of 6	855	81.1	8	103.1
2000	S1	6 of 6	1660	65.9	4.4	99.4

Table B 8.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (mg/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	15.8	1.76	10.9	101
45	L QC	6 of 6	46.5	5.84	12.4	103.2
800	M QC	6 of 6	851	47.5	5.4	106.4
1600	H QC	6 of 6	1810	158	8.7	113.2

Citalopram: Serum Validation 1, Day 1

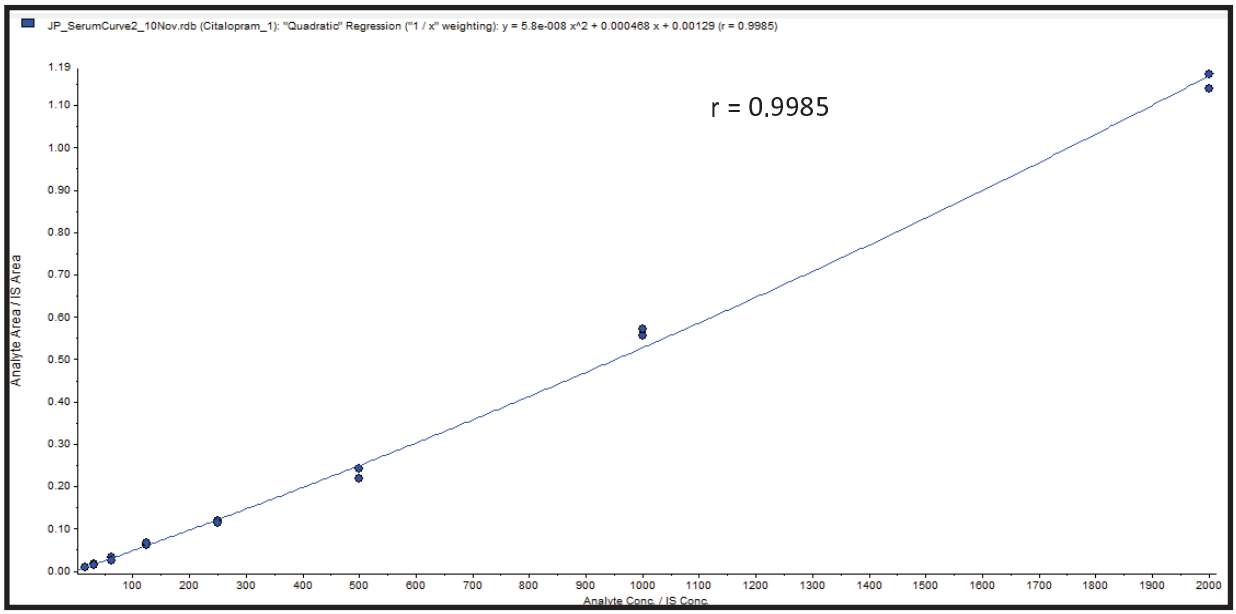


Figure B 7. Representative calibration curve for Citalopram: Validation 1, Day 1.

Table B 9.1. Citalopram Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	14	2.71	19.4	89.6
31.3	S7	2 of 2	37.7	2.54	6.7	120.5
62.5	S6	2 of 2	56.1	0.77	1.4	89.8
125	S5	2 of 2	123	0.48	0.4	98.4
250	S4	2 of 2	252	12.9	5.1	100.9
500	S3	1 of 1	514	N/A	N/A	102.9
1000	S2	2 of 2	992	71.2	7.2	99.2
2000	S1	2 of 2	2000	66.8	3.3	100.1

Table B 9.2. Summary of Citalopram intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	13.2	2.12	16	84.8
45	L QC	2 of 2	49.2	5.65	11.5	109.4
800	M QC	2 of 2	961	20.4	2.1	120.1
1600	H QC	2 of 2	2020	53.1	2.6	126

Citalopram: Serum Validation 2, Day 2

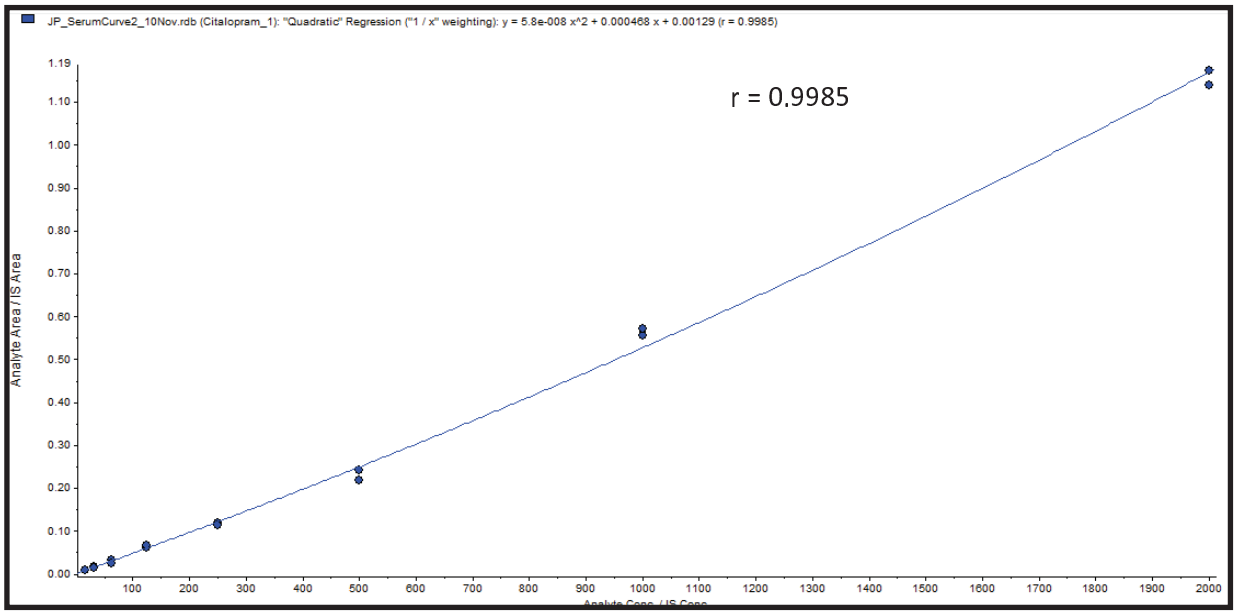


Figure B 8. Representative calibration curve for Citalopram: Validation 2, Day 2.

Table B 10.1. Citalopram Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.7	0.26	1.5	106.9
31.3	S7	2 of 2	32	2.83	8.9	102.2
62.5	S6	2 of 2	58.1	12	20.7	92.9
125	S5	2 of 2	131	7.07	5.4	104.5
250	S4	2 of 2	239	8.72	3.6	95.7
500	S3	2 of 2	462	30.4	6.6	92.4
1000	S2	2 of 2	1060	19	1.8	106.2
2000	S1	2 of 2	1980	33.5	1.7	99.1

Table B 10.2. Summary of Citalopram intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	Number Of Values Used	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	14.2	1.34	9.5	90.9
45	L QC	2 of 2	50.3	2.89	5.7	111.8
800	M QC	2 of 2	863	20.3	2.4	107.9
1600	H QC	2 of 2	1700	195	11.4	106.4

Citalopram: Serum Validation 3, Day 2

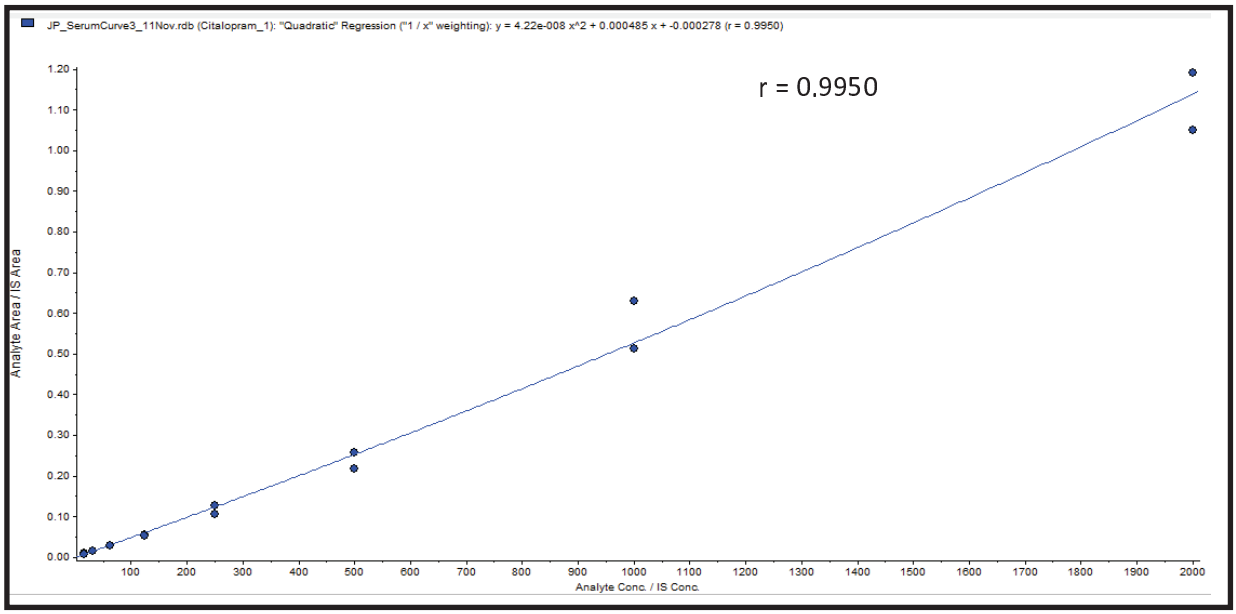


Figure B 9. Representative calibration curve for Citalopram: Validation 3, Day 2.

Table B 11.1. Citalopram Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.6	2.88	18.5	100
31.3	S7	2 of 2	30.6	0.69	2.3	97.9
62.5	S6	2 of 2	68.1	5.19	7.6	108.9
125	S5	2 of 2	125	14.4	11.5	100
250	S4	2 of 2	237	8.25	3.5	94.9
500	S3	2 of 2	460	13.5	2.9	92.1
1000	S2	2 of 2	1070	235	21.9	107.4
2000	S1	2 of 2	1970	168	8.5	98.7

Table B 11.2. Summary of Citalopram intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	15.7	0.25	1.6	100.4
45	L QC	2 of 2	48	10.8	22.4	106.8
800	M QC	2 of 2	803	1.33	0.2	100.4
1600	H QC	2 of 2	1780	73.2	4.1	111.5

Table B 12.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	15.4	1.95	13.1	98.8
31.3	S7	6 of 6	33.4	2.02	5.9	106.9
62.5	S6	6 of 6	60.8	6	9.9	97.2
125	S5	6 of 6	126	7.31	5.8	101
250	S4	6 of 6	238	8.48	3.6	95.3
500	S3	5 of 5	392	18.9	4.9	95.2
1000	S2	6 of 6	883	127	11.8	105.5
2000	S1	6 of 6	1650	90.8	5.8	99

Table B 12.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	14.4	1.24	9	92
45	L QC	6 of 6	49.2	6.43	13.2	109.3
800	M QC	6 of 6	876	14	1.5	109.5
1600	H QC	6 of 6	1830	107	6.1	114.6

Alprazolam: Serum Validation 1, Day1

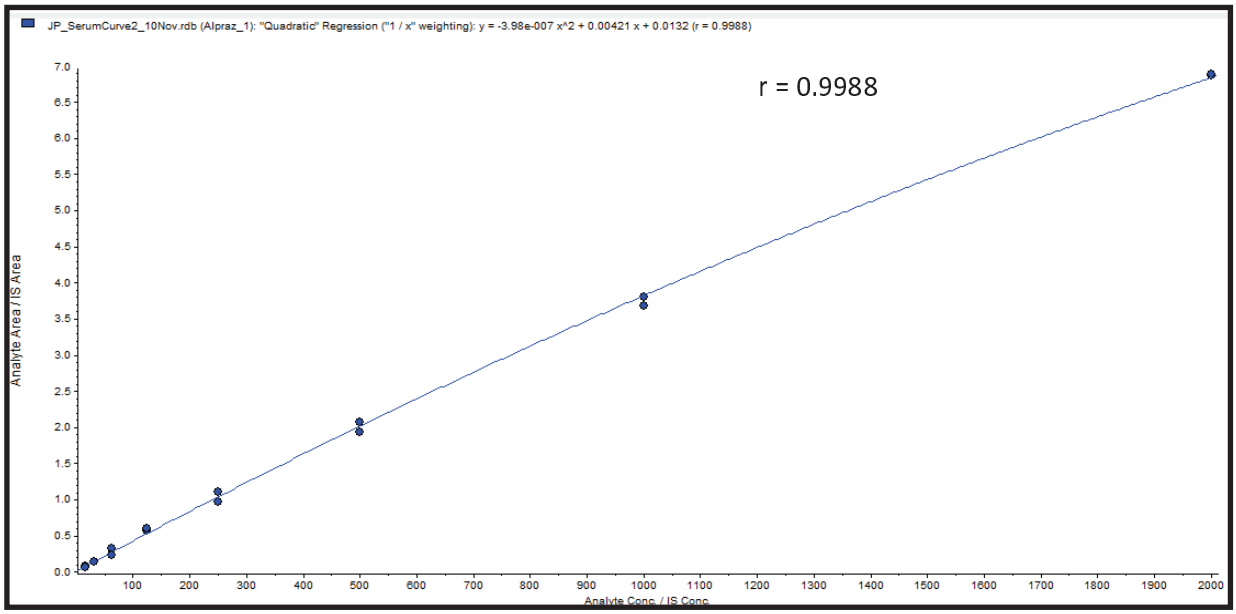


Figure B 10. Representative calibration curve for Alprazolam: Validation 1, Day 1.

Table B 13.1. Alprazolam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (mg/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.1	1.19	9.1	83.9
31.3	S7	2 of 2	36.9	1.31	3.5	117.8
62.5	S6	2 of 2	58.4	8.39	14.4	93.4
125	S5	2 of 2	124	11.7	9.4	99.4
250	S4	2 of 2	276	18.1	6.6	110.3
500	S3	1 of 1	464	N/A	N/A	92.7
1000	S2	2 of 2	982	89	9.1	98.2
2000	S1	2 of 2	2020	231	11.5	100.9

Table B 13.2. Summary of Alprazolam intra-validation quality control standards – Validation 1

Expected Concentration	Sample Name	Number Of Values Used	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	15.6	1.63	10.4	100
45	L QC	2 of 2	49.3	1.64	3.3	109.5
800	M QC	2 of 2	914	161	17.7	114.3
1600	H QC	2 of 2	1910	139	7.3	119.4

Alprazolam: Serum Validation 2, Day 2

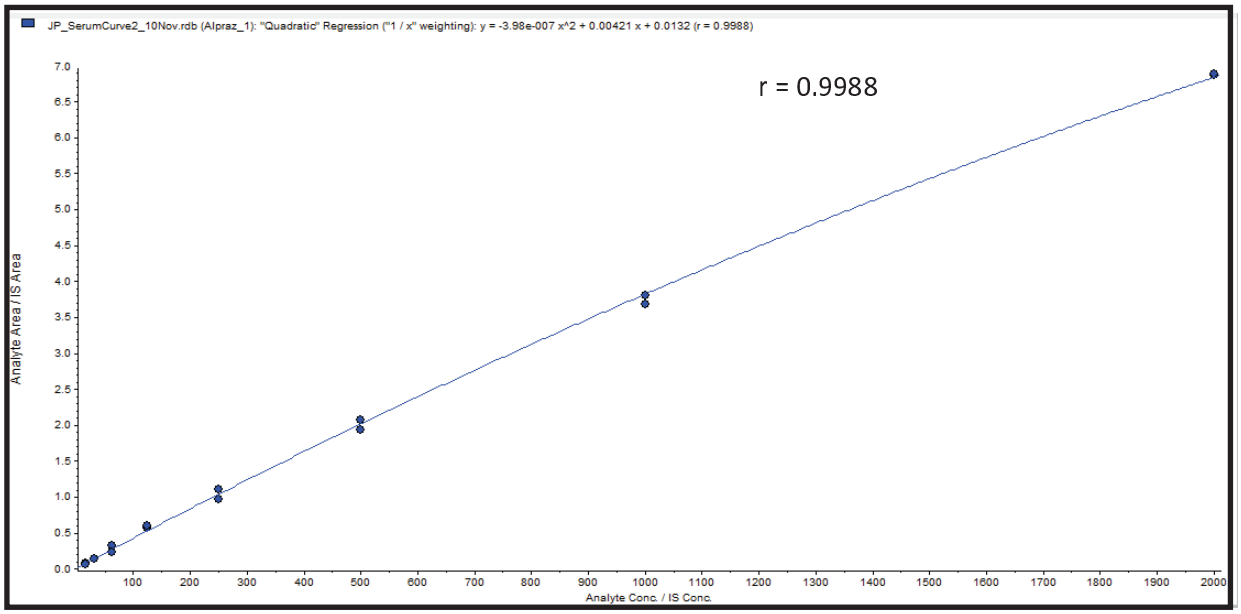


Figure B 11. Representative calibration curve for Alprazolam: Validation 2, Day 2.

Table B 14.1. Alprazolam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	Number Of Values Used	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.7	1.89	13.8	87.6
31.3	S7	2 of 2	31.4	1.69	5.4	100.2
62.5	S6	2 of 2	64.7	14.6	22.6	103.5
125	S5	2 of 2	140	4.63	3.3	111.6
250	S4	2 of 2	249	23.4	9.4	99.6
500	S3	2 of 2	497	26.2	5.3	99.3
1000	S2	2 of 2	975	25.1	2.6	97.5
2000	S1	2 of 2	2020	3.13	0.2	100.8

Table B 14.2. Summary of Alprazolam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	14.6	0.49	3.3	93.9
45	L QC	2 of 2	46.7	2.98	6.4	103.8
800	M QC	2 of 2	800	9.81	1.2	100
1600	H QC	2 of 2	1550	298	19.2	96.9

Alprazolam: Serum Validation 3, Day 2

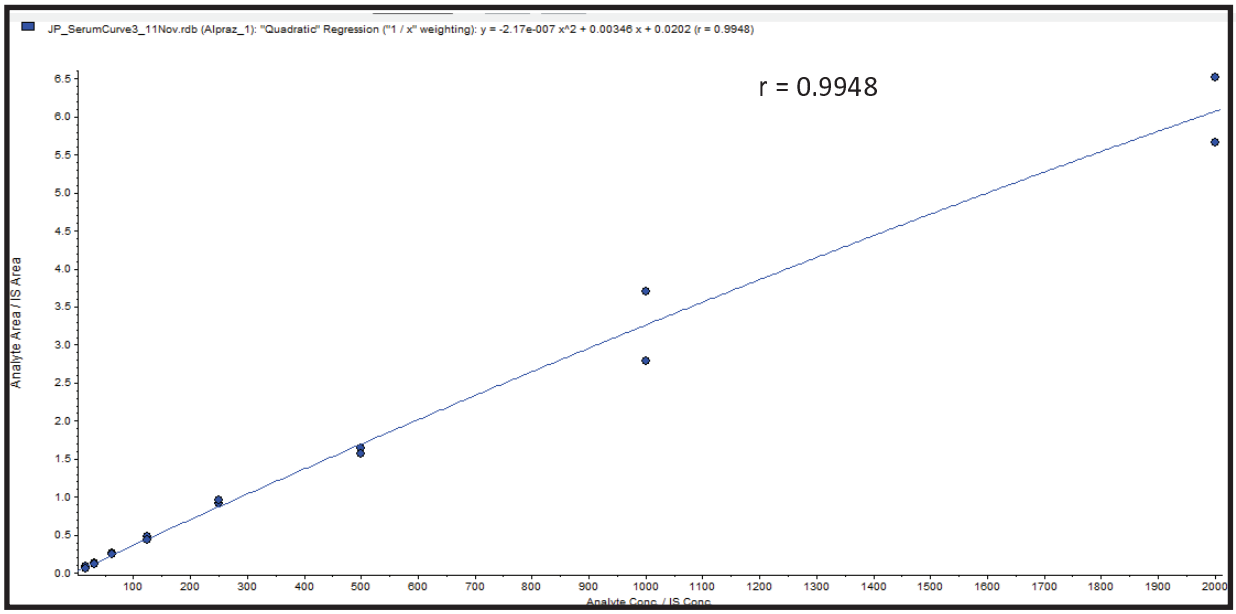


Figure B 12. Representative calibration curve for Alprazolam: Validation 3, Day 2.

Table B 15.1. Alprazolam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	14.8	5.27	35.6	94.7
31.3	S7	2 of 2	28.9	3.51	12.1	92.4
62.5	S6	2 of 2	67.9	4.96	7.3	108.6
125	S5	2 of 2	128	8.6	6.7	102
250	S4	2 of 2	268	9.54	3.6	107.4
500	S3	2 of 2	474	15	3.2	94.8
1000	S2	2 of 2	998	214	21.5	99.8
2000	S1	2 of 2	2010	235	11.7	100.5

Table B 15.2. Summary of Alprazolam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	11.3	2.11	18.6	72.7
45	L QC	2 of 2	41.6	6.43	15.5	92.3
800	M QC	2 of 2	831	13.1	1.6	103.9
1600	H QC	2 of 2	1870	62.9	3.4	116.6

Table B 16.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	13.8	2.78	19.5	88.8
31.3	S7	6 of 6	32.4	2.17	7	103.4
62.5	S6	6 of 6	63.7	9.32	14.8	101.8
125	S5	6 of 6	130	8.3	6.5	104.3
250	S4	6 of 6	259	16.5	6.5	103.5
500	S3	5 of 5	415	19.8	5	101.5
1000	S2	6 of 6	812	120	12	96.7
2000	S1	6 of 6	1670	109	7	99.8

Table B 16.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	13.9	1.41	10.8	88.9
45	L QC	6 of 6	45.8	3.68	8.4	101.9
800	M QC	6 of 6	848	61.4	6.8	106
1600	H QC	6 of 6	1780	167	10	111

Clonazepam: Serum Validation 1, Day1

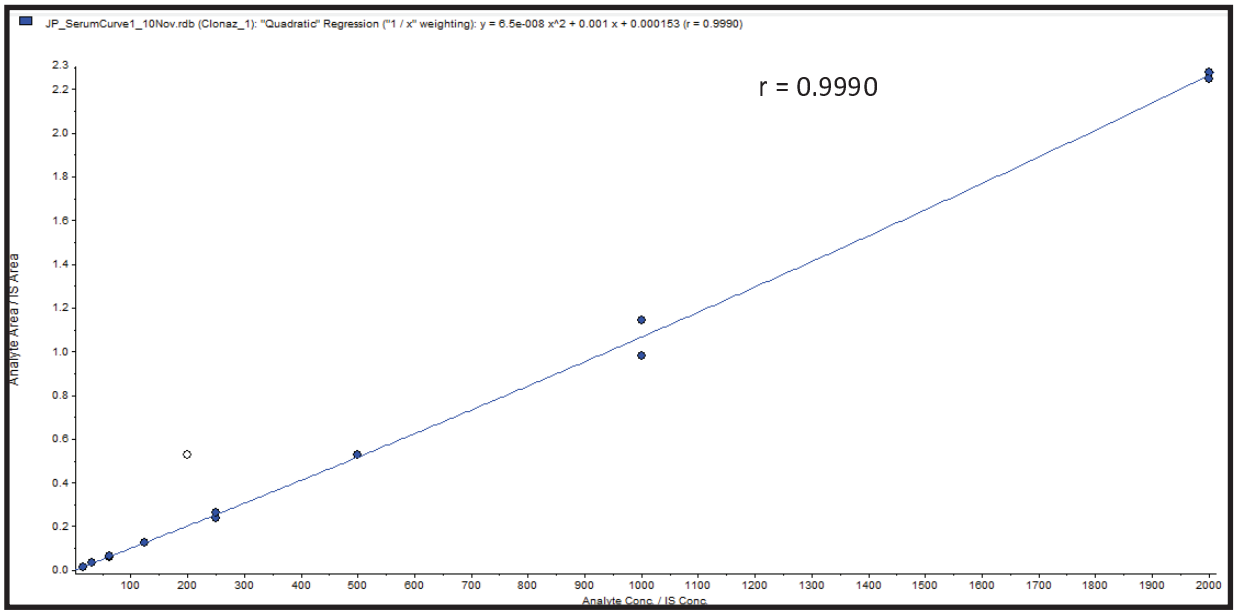


Figure B 13. Representative calibration curve for Clonazepam: Validation 1, Day 1.

Table B 17.1. Clonazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	14.1	0.76	5.4	90.5
31.3	S7	2 of 2	34	2.37	7	108.8
62.5	S6	2 of 2	64.5	3.51	5.4	103.1
125	S5	2 of 2	123	0.3	0.2	98.3
250	S4	2 of 2	246	14.1	5.7	98.6
500	S3	1 of 1	509	N/A	N/A	101.9
1000	S2	2 of 2	997	100	10.1	99.7
2000	S1	2 of 2	2000	16.1	0.8	100

Table B 17.2. Summary of Clonazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	16.4	2.59	15.8	105.1
45	L QC	2 of 2	49.8	2.21	4.4	110.8
800	M QC	2 of 2	985	109	11.1	123.1
1600	H QC	2 of 2	2070	34.9	1.7	129.2

Clonazepam: Serum Validation 2, Day 2

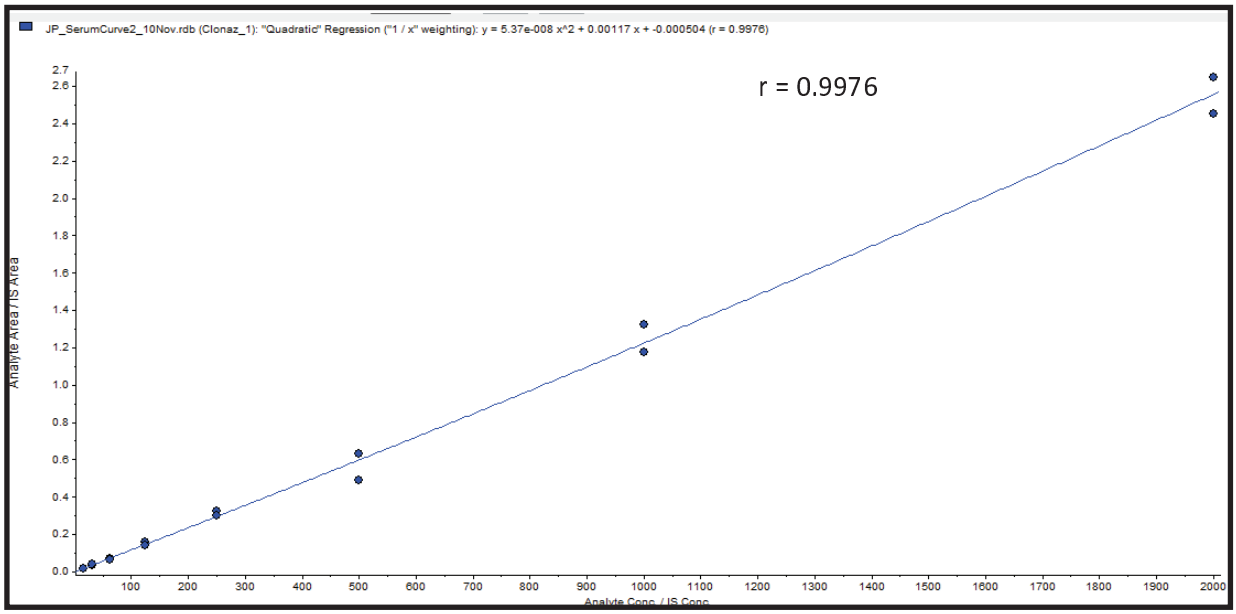


Figure B 14. Representative calibration curve for Clonazepam: Validation 2, Day 2.

Table B 18.1. Clonazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.2	0.93	6.1	97.1
31.3	S7	2 of 2	32.8	1.66	5.1	104.8
62.5	S6	2 of 2	59.4	4.1	6.9	95
125	S5	2 of 2	126	11.4	9	101.2
250	S4	2 of 2	265	14.4	5.4	106
500	S3	2 of 2	470	80.9	17.2	94
1000	S2	2 of 2	1020	82.2	8.1	101.9
2000	S1	2 of 2	2000	99.1	5	99.8

Table B 18.2. Summary of Clonazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	13.5	0.5	3.7	86.4
45	L QC	2 of 2	43.5	2.66	6.1	96.6
800	M QC	2 of 2	856	24.6	2.9	107.1
1600	H QC	2 of 2	1680	177	10.5	105.2

Clonazepam: Serum Validation 3, Day 2

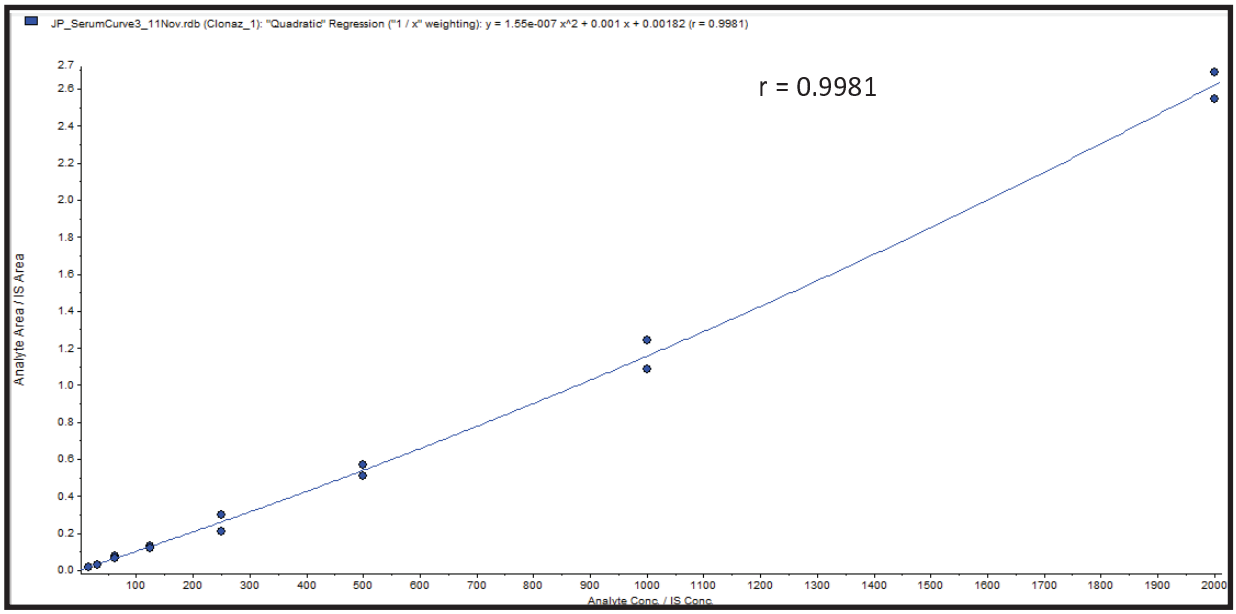


Figure B 15. Representative calibration curve for Clonazepam: Validation 3, Day 2.

Table B 19.1. Clonazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.8	0.8	5.1	101
31.3	S7	2 of 2	29.2	1.43	4.9	93.3
62.5	S6	2 of 2	68	6.99	10.3	108.8
125	S5	2 of 2	123	9.32	7.6	98.6
250	S4	2 of 2	244	59	24.2	97.5
500	S3	2 of 2	500	36.5	7.3	100
1000	S2	2 of 2	1010	86.3	8.6	100.5
2000	S1	2 of 2	2000	62.6	3.1	99.9

Table B 19.2. Summary of Clonazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	17.5	1.55	8.9	112
45	L QC	2 of 2	48.7	0.72	1.5	108.3
800	M QC	2 of 2	838	71.2	8.5	104.8
1600	H QC	2 of 2	1700	45	2.7	106.2

Table B 20.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	15	0.83	5.5	96.2
31.3	S7	6 of 6	32	1.82	5.6	102.3
62.5	S6	6 of 6	63.9	4.86	7.5	102.3
125	S5	6 of 6	124	7.01	5.6	99.4
250	S4	6 of 6	254	36.7	14.8	101.8
500	S3	5 of 5	405	43.8	10.1	97.5
1000	S2	6 of 6	844	84.2	8.3	101.4
2000	S1	6 of 6	1660	87.3	6.1	99.8

Table B 20.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	15.8	1.55	9.5	101.2
45	L QC	6 of 6	47.4	1.86	4	105.2
800	M QC	6 of 6	893	68.4	7.5	111.6
1600	H QC	6 of 6	1820	85.6	4.9	113.5

Diazepam: Serum Validation 1, Day1

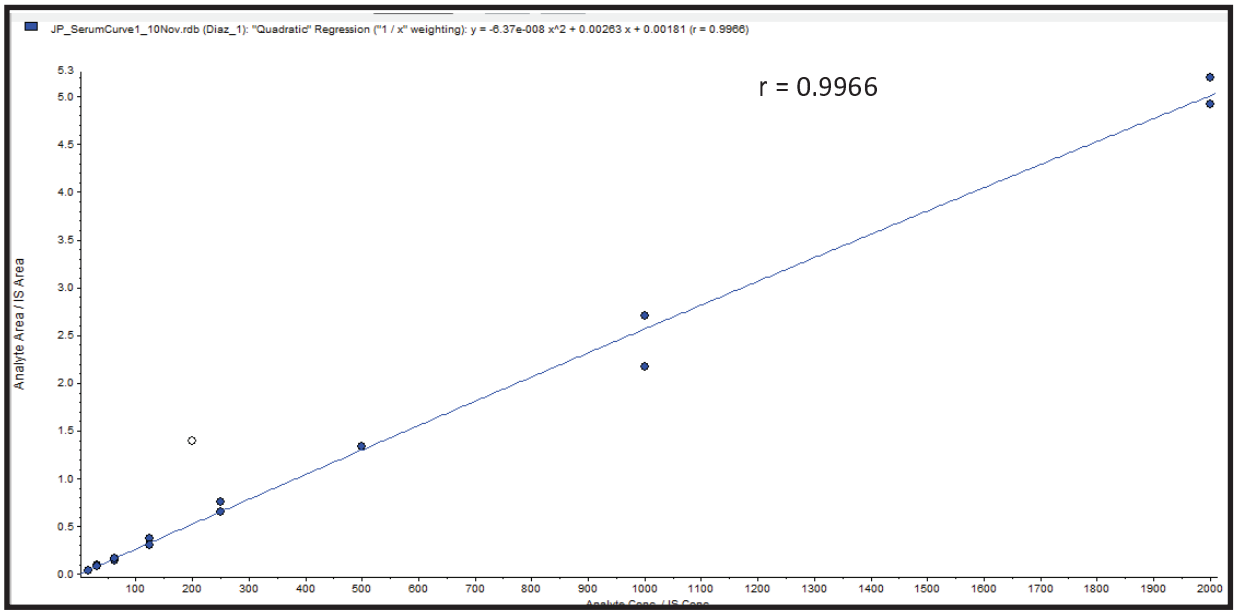


Figure B 16. Representative calibration curve for Diazepam: Validation 1, Day 1.

Table B 21.1. Diazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.7	0.07	0.5	87.9
31.3	S7	2 of 2	34.5	1.75	5.1	110.3
62.5	S6	2 of 2	57.9	5.93	10.2	92.7
125	S5	2 of 2	129	18.2	14.1	103.4
250	S4	2 of 2	271	28.8	10.6	108.3
500	S3	1 of 1	516	N/A	N/A	103.2
1000	S2	2 of 2	949	151	16	94.9
2000	S1	2 of 2	2020	83	4.1	101.1

Table B 21.2. Summary of Diazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	16.3	0.97	6	104.6
45	L QC	2 of 2	45.3	3.17	7	100.6
800	M QC	2 of 2	832	89.7	10.8	104
1600	H QC	2 of 2	1720	215	12.5	107.6

Diazepam: Serum Validation 2, Day 2

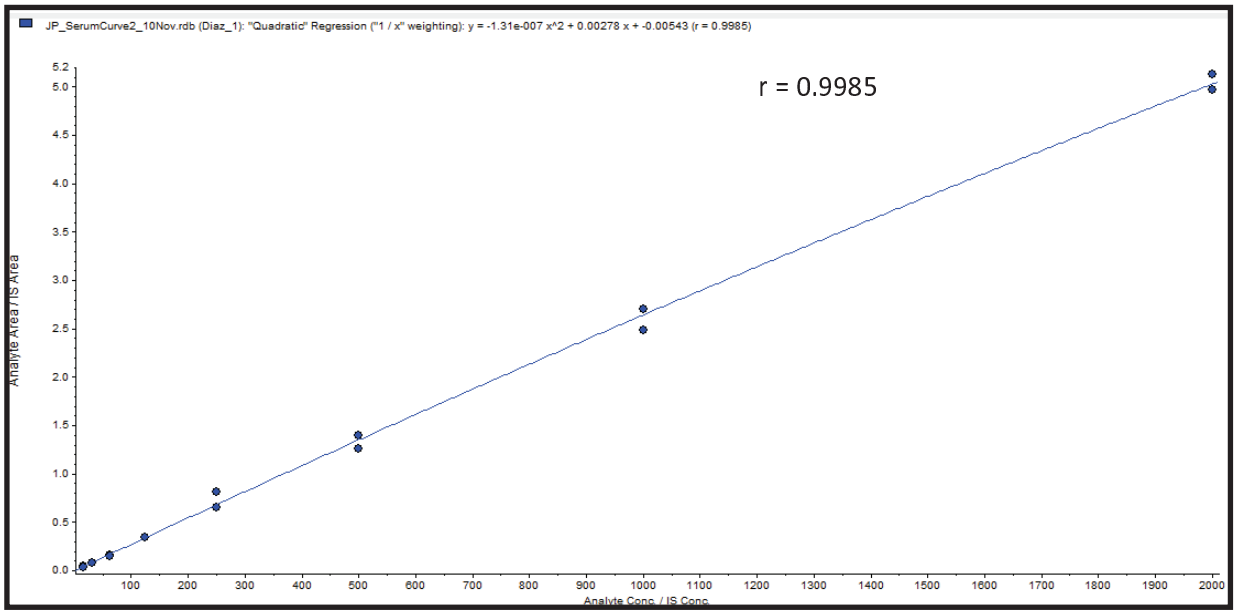


Figure B 17. Representative calibration curve for Diazepam: Validation 2, Day 2.

Table B 22.1. Diazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.4	1.81	11.8	98.6
31.3	S7	2 of 2	31.8	0.62	1.9	101.7
62.5	S6	2 of 2	58.2	5.37	9.2	93
125	S5	2 of 2	127	0.8	0.6	101.9
250	S4	2 of 2	269	40.5	15	107.8
500	S3	2 of 2	492	35.8	7.3	98.4
1000	S2	2 of 2	980	60.1	6.1	98
2000	S1	2 of 2	2010	48.8	2.4	100.5

Table B 22.2. Summary of Diazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	17.4	1.34	7.7	111.5
45	L QC	2 of 2	40.2	4.58	11.4	89.4
800	M QC	2 of 2	816	35.9	4.4	102
1600	H QC	2 of 2	1650	209	12.7	103.4

Diazepam: Serum Validation 3, Day 2

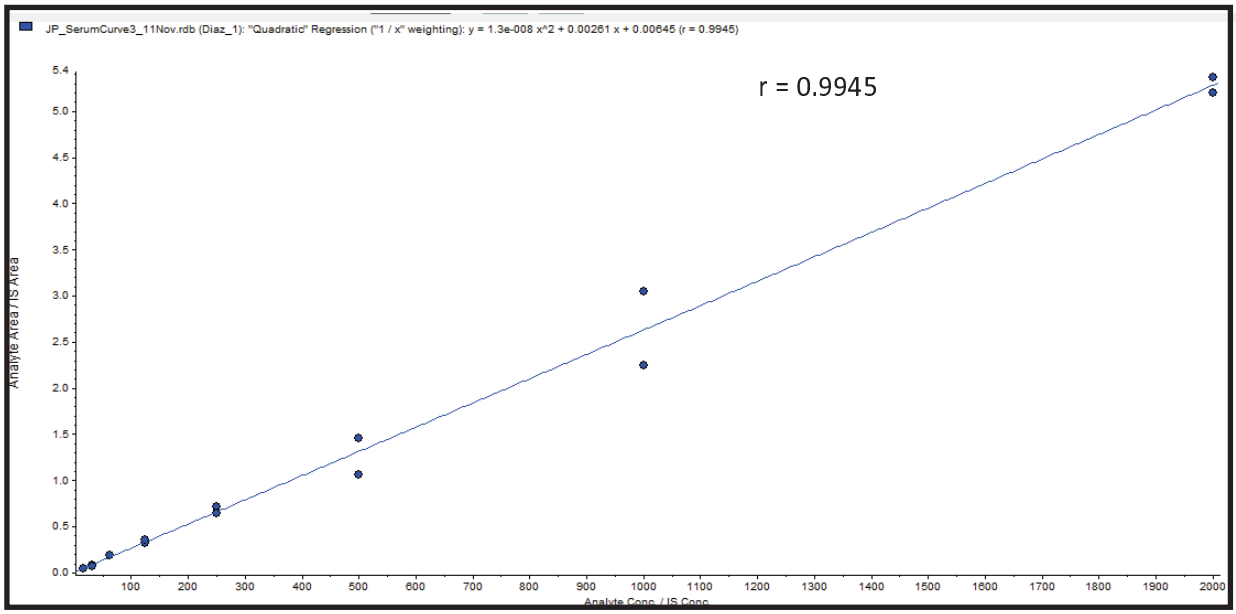


Figure B 18. Representative calibration curve for Diazepam: Validation 3, Day 2.

Table B 23.1. Diazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.2	0.24	1.6	97.3
31.3	S7	2 of 2	28	2.53	9	89.4
62.5	S6	2 of 2	69.6	1.34	1.9	111.4
125	S5	2 of 2	128	9.94	7.8	102.4
250	S4	2 of 2	257	20.5	8	102.8
500	S3	2 of 2	479	104	21.7	95.8
1000	S2	2 of 2	1010	216	21.5	100.7
2000	S1	2 of 2	2000	45.7	2.3	100

Table B 23.2. Summary of Diazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	13.8	0.98	7.1	88.5
45	L QC	2 of 2	38.3	1.87	4.9	85.1
800	M QC	2 of 2	770	21.2	2.8	96.2
1600	H QC	2 of 2	1730	29.3	1.7	108

Table B 24.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	14.8	0.71	4.6	94.6
31.3	S7	6 of 6	31.5	1.63	5.3	100.5
62.5	S6	6 of 6	61.9	4.21	7.1	99
125	S5	6 of 6	128	9.64	7.5	102.6
250	S4	6 of 6	263	30.5	11.5	105.3
500	S3	5 of 5	414	56.2	13.2	100.8
1000	S2	6 of 6	834	138	13.8	100.6
2000	S1	6 of 6	1650	82	6.9	98.5

Table B 24.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	15.8	1.1	6.9	101.5
45	L QC	6 of 6	41.3	3.21	7.8	91.7
800	M QC	6 of 6	806	48.9	6	100.7
1600	H QC	6 of 6	1700	151	8.9	106.4

Flunitrazepam: Serum Validation 1, Day1

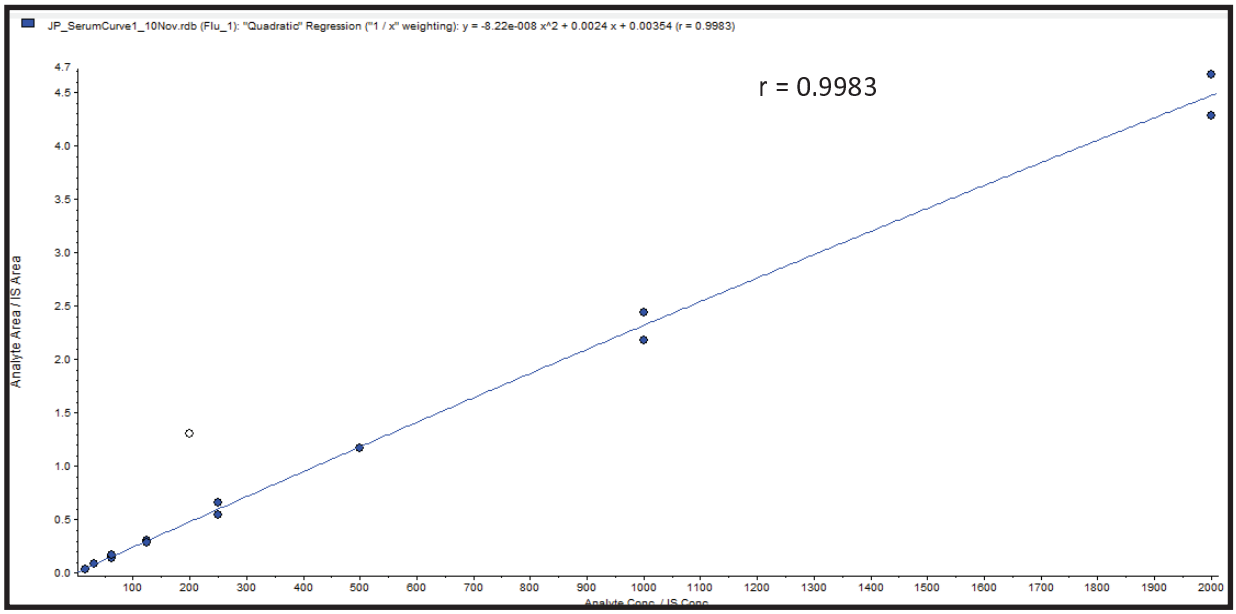


Figure B 19. Representative calibration curve for Flunitrazepam: Validation 1, Day 1.

Table B 25.1. Flunitrazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.7	1.47	10.7	87.9
31.3	S7	2 of 2	35	0.64	1.8	111.8
62.5	S6	2 of 2	63.5	6.51	10.2	101.6
125	S5	2 of 2	122	6.55	5.3	98
250	S4	2 of 2	253	35	13.8	101.4
500	S3	1 of 1	496	N/A	N/A	99.1
1000	S2	2 of 2	997	82.7	8.3	99.7
2000	S1	2 of 2	2000	132	6.6	100.1

Table B 25.2. Summary of Flunitrazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	16.8	2.03	12.1	107.7
45	L QC	2 of 2	47.7	0.66	1.4	105.9
800	M QC	2 of 2	850	16.5	1.9	106.2
1600	H QC	2 of 2	1850	133	7.2	115.8

Flunitrazepam: Serum Validation 2, Day 2

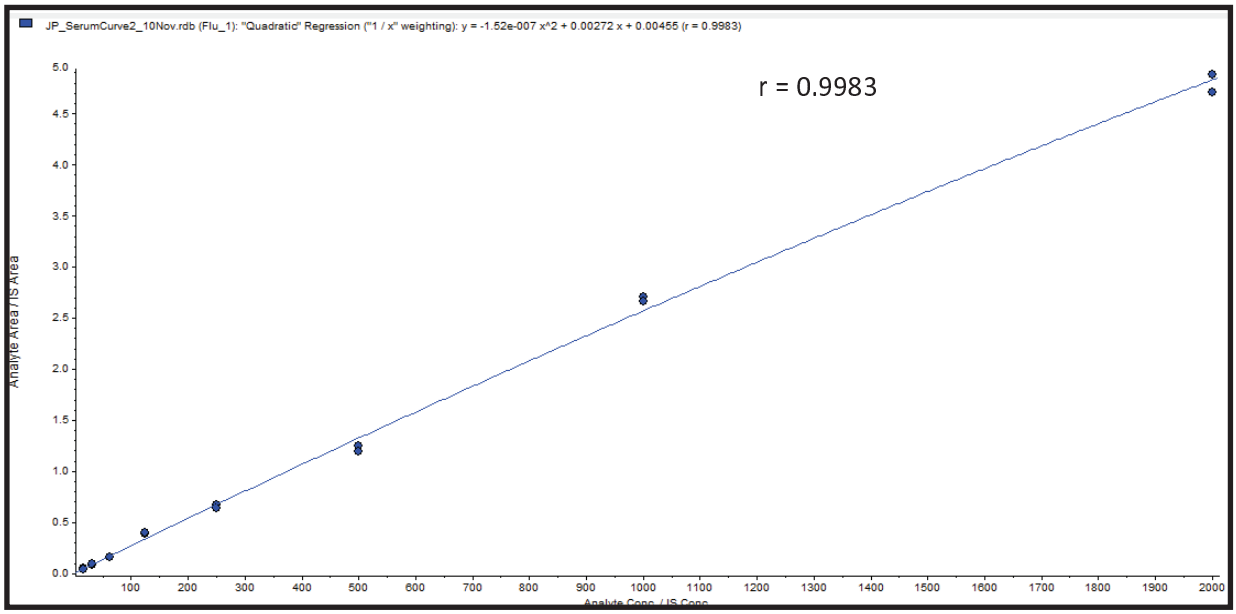


Figure B 20. Representative calibration curve for Flunitrazepam: Validation 2, Day 2.

Table B 26.1. Flunitrazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.6	3.66	23.5	99.9
31.3	S7	2 of 2	31	1.42	4.6	99.2
62.5	S6	2 of 2	57.3	2.2	3.8	91.8
125	S5	2 of 2	145	2.64	1.8	115.8
250	S4	2 of 2	244	9.4	3.9	97.5
500	S3	2 of 2	459	12.6	2.8	91.9
1000	S2	2 of 2	1050	10.2	1	104.8
2000	S1	2 of 2	1980	57.2	2.9	99.2

Table B 26.2. Summary of Flunitrazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	13.6	0.38	2.8	87.2
45	L QC	2 of 2	43.8	8.78	20	97.4
800	M QC	2 of 2	768	30.3	3.9	96
1600	H QC	2 of 2	1560	16	1	97.7

Flunitrazepam: Serum Validation 3, Day 2

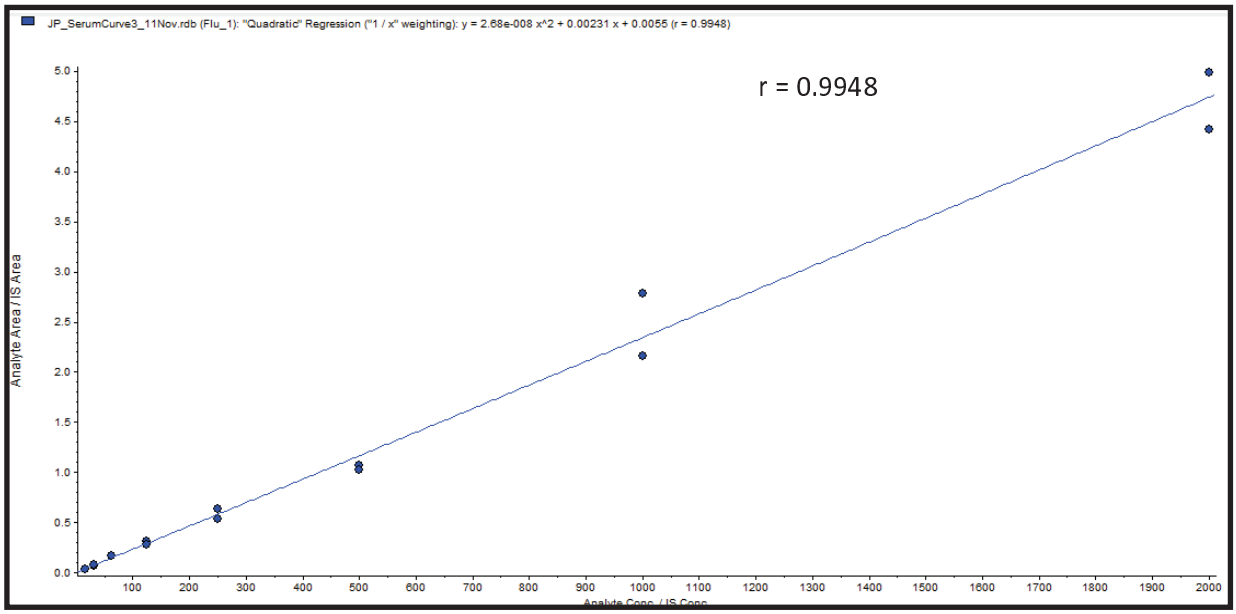


Figure B 21. Representative calibration curve for Flunitrazepam: Validation 3, Day 2.

Table B 27.1. Flunitrazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	14.5	1.28	8.8	93.2
31.3	S7	2 of 2	29.9	2.62	8.8	95.5
62.5	S6	2 of 2	71.2	0.31	0.4	113.9
125	S5	2 of 2	127	9.58	7.6	101.3
250	S4	2 of 2	253	29.1	11.5	101.1
500	S3	2 of 2	450	15.3	3.4	90.1
1000	S2	2 of 2	1050	185	17.6	105.4
2000	S1	2 of 2	1980	166	8.4	99.2

Table B 27.2. Summary of Flunitrazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	13.8	0.18	1.3	88.8
45	L QC	2 of 2	50	0.08	0.2	111
800	M QC	2 of 2	768	12.3	1.6	96
1600	H QC	2 of 2	1750	82.2	4.7	109.5

Table B 28.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	14.6	2.14	14.3	93.7
31.3	S7	6 of 6	32	1.56	5.1	102.2
62.5	S6	6 of 6	64	3.01	4.8	102.4
125	S5	6 of 6	131	6.26	4.9	105
250	S4	6 of 6	248	19.3	7.7	99.3
500	S3	5 of 5	388	21	6.7	94.4
1000	S2	6 of 6	866	97.8	9.3	103.1
2000	S1	6 of 6	1660	102	6.5	99.4

Table B 28.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	14.8	0.86	5.4	94.6
45	L QC	6 of 6	47.1	3.18	7.2	104.8
800	M QC	6 of 6	795	19.7	2.5	99.4
1600	H QC	6 of 6	1720	77.1	4.3	107.6

Lorazepam: Serum Validation 1, Day1

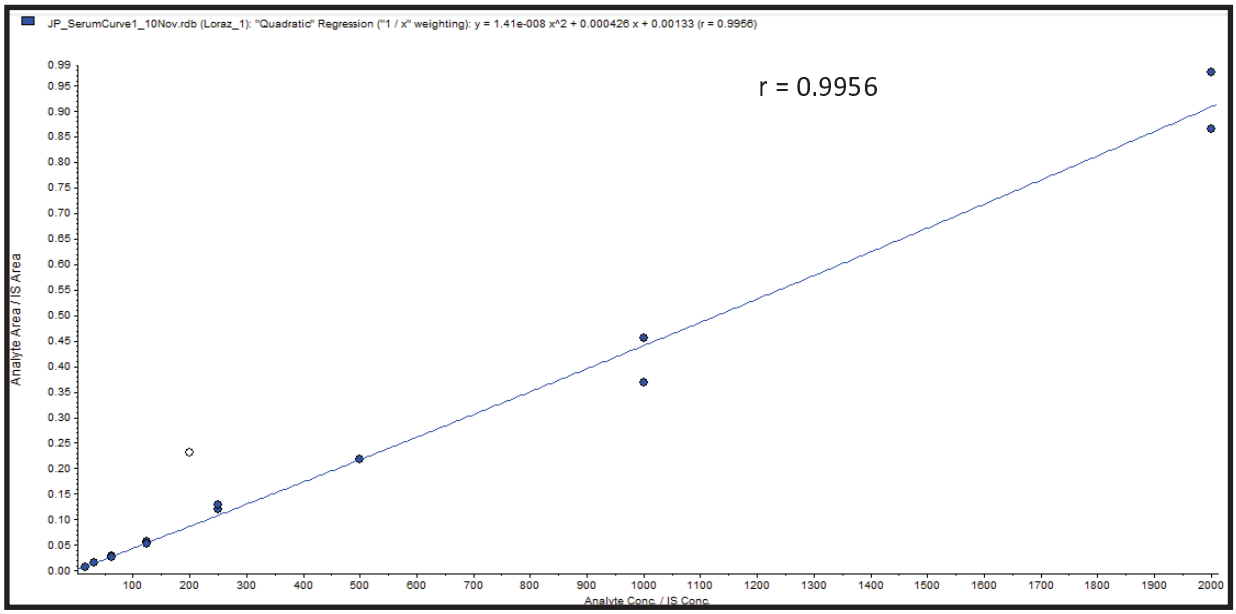


Figure B 22. Representative calibration curve for Lorazepam: Validation 1, Day 1.

Table B 29.1. Lorazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13	0.1	0.7	83.4
31.3	S7	2 of 2	33.8	2.19	6.5	107.9
62.5	S6	2 of 2	61.1	3.42	5.6	97.7
125	S5	2 of 2	125	7.43	5.9	100.2
250	S4	2 of 2	290	14.2	4.9	115.9
500	S3	1 of 1	501	N/A	N/A	100.2
1000	S2	2 of 2	935	136	14.6	93.5
2000	S1	2 of 2	2030	163	8	101.3

Table B 29.2. Summary of Lorazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	14.6	1.34	9.2	93.8
45	L QC	2 of 2	54	4.24	7.9	120.1
800	M QC	2 of 2	928	71.5	7.7	116
1600	H QC	2 of 2	1950	36.1	1.9	121.7

Lorazepam: Serum Validation 2, Day 2

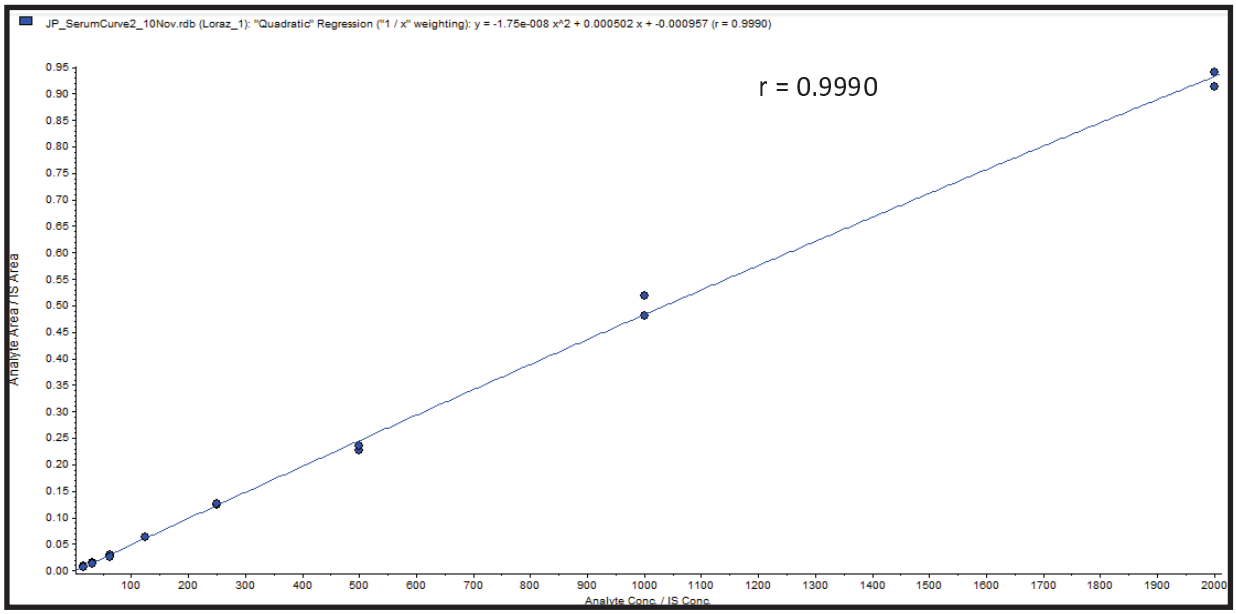


Figure B 23. Representative calibration curve for Lorazepam: Validation 2, Day 2.

Table B 30.1. Lorazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.4	2.03	12.4	105.1
31.3	S7	2 of 2	32	3.11	9.7	102.2
62.5	S6	2 of 2	55.7	6.14	11	89.1
125	S5	2 of 2	130	0.63	0.5	104.3
250	S4	2 of 2	255	2.61	1	102.2
500	S3	2 of 2	472	11.4	2.4	94.3
1000	S2	2 of 2	1030	57.8	5.6	103.5
2000	S1	2 of 2	1990	43.4	2.2	99.4

Table B 30.2. Summary of Lorazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	16.5	0.08	0.5	105.7
45	L QC	2 of 2	41.8	3.92	9.4	93
800	M QC	2 of 2	799	71.8	9	99.8
1600	H QC	2 of 2	1610	173	10.7	100.6

Lorazepam: Serum Validation 3, Day 2

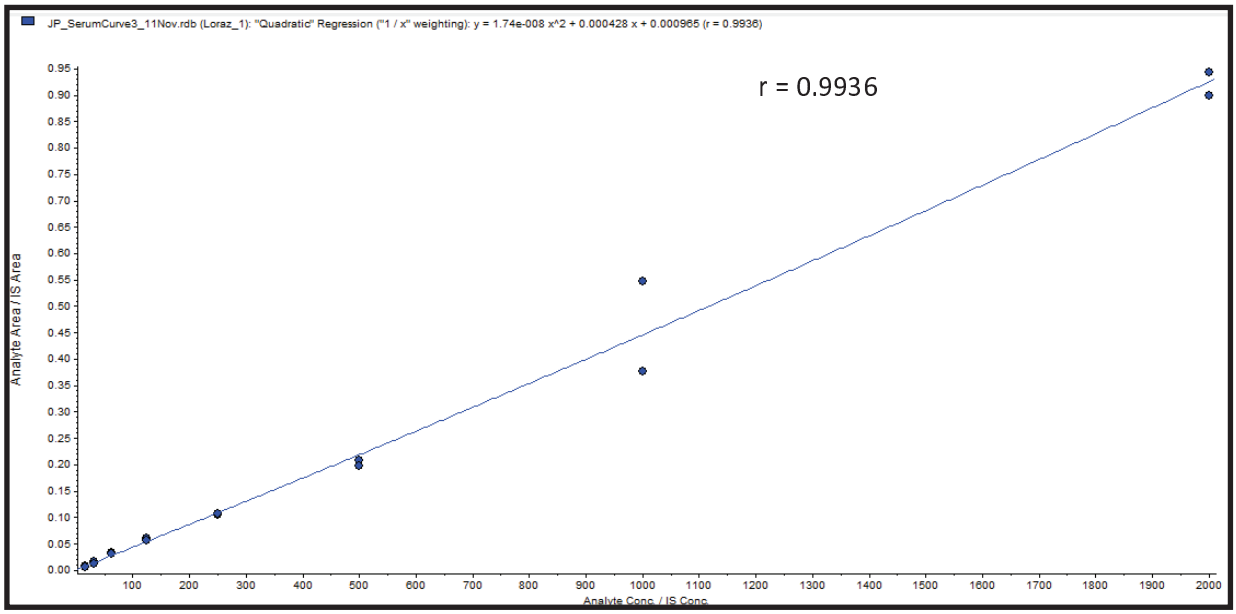


Figure B 24. Representative calibration curve for Lorazepam: Validation 3, Day 2.

Table B 31.1. Lorazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13	2.28	17.5	83.3
31.3	S7	2 of 2	31.3	4.8	15.3	100.1
62.5	S6	2 of 2	72.3	5.22	7.2	115.8
125	S5	2 of 2	135	8.61	6.4	107.9
250	S4	2 of 2	242	2.33	1	97
500	S3	2 of 2	463	17	3.7	92.6
1000	S2	2 of 2	1030	263	25.4	103.4
2000	S1	2 of 2	1990	62.3	3.1	99.6

Table B 31.2. Summary of Lorazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	14.4	0.4	2.8	92.1
45	L QC	2 of 2	46.4	4.06	8.8	103
800	M QC	2 of 2	722	17.1	2.4	90.2
1600	H QC	2 of 2	1790	19.1	1.1	112.2

Table B 32.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	14.1	1.47	10.2	90.6
31.3	S7	6 of 6	32.4	3.37	10.5	103.4
62.5	S6	6 of 6	63	4.92	7.9	100.8
125	S5	6 of 6	130	5.56	4.3	104.1
250	S4	6 of 6	249	2.47	1	99.6
500	S3	5 of 5	408	14.2	3.7	100.9
1000	S2	6 of 6	857	160	15.5	102.4
2000	S1	6 of 6	1640	80.7	6.6	97.5

Table B 32.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	15.2	0.61	4.1	97.2
45	L QC	6 of 6	47.4	4.07	8.7	105.4
800	M QC	6 of 6	816	53.5	6.4	102
1600	H QC	6 of 6	1780	75.9	4.5	111.5

Nitrazepam: Nitrazepam Validation 1, Day1

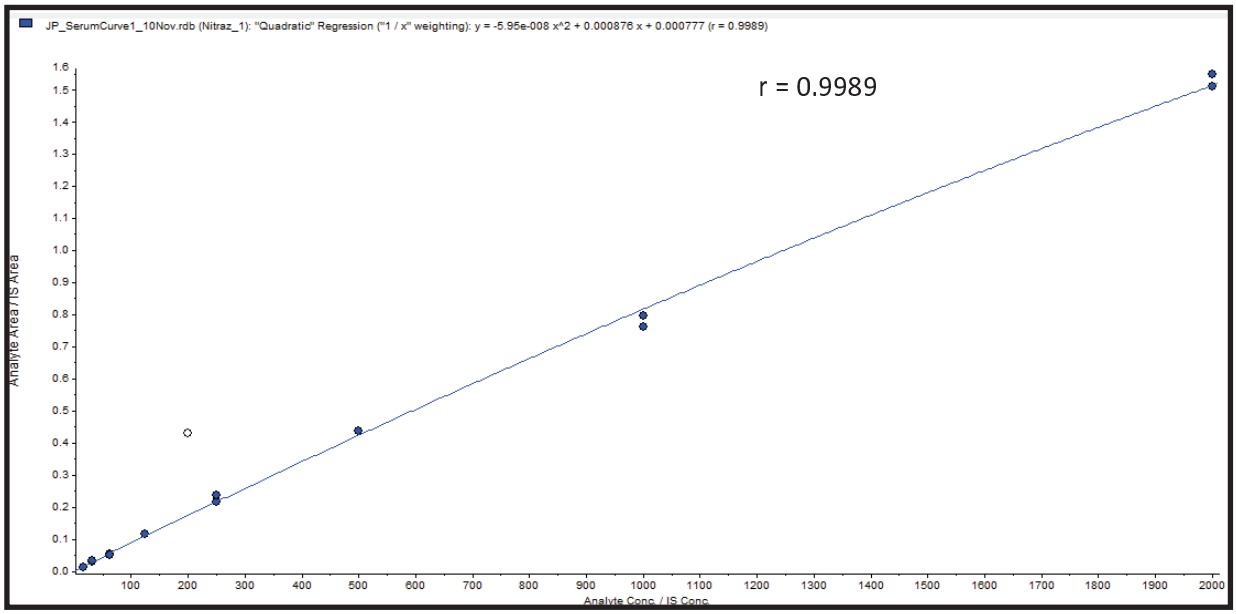


Figure B 25. Representative calibration curve for Nitrazepam: Validation 1, Day 1.

Table B 33.1. Nitrazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.2	0.49	3.7	84.5
31.3	S7	2 of 2	35.1	0.98	2.8	112
62.5	S6	2 of 2	58.4	2.35	4	93.4
125	S5	2 of 2	134	0.36	0.3	107.3
250	S4	2 of 2	263	19.4	7.4	105.2
500	S3	1 of 1	516	N/A	N/A	103.2
1000	S2	2 of 2	949	31.1	3.3	94.9
2000	S1	2 of 2	2030	43.4	2.1	101.3

Table B 33.2. Summary of Nitrazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	14.8	1.47	9.9	95.1
45	L QC	2 of 2	57.1	2.93	5.1	126.8
800	M QC	2 of 2	961	1.66	0.2	120.2
1600	H QC	2 of 2	2200	202	9.1	137.8

Nitrazepam: Serum Validation 2, Day 2

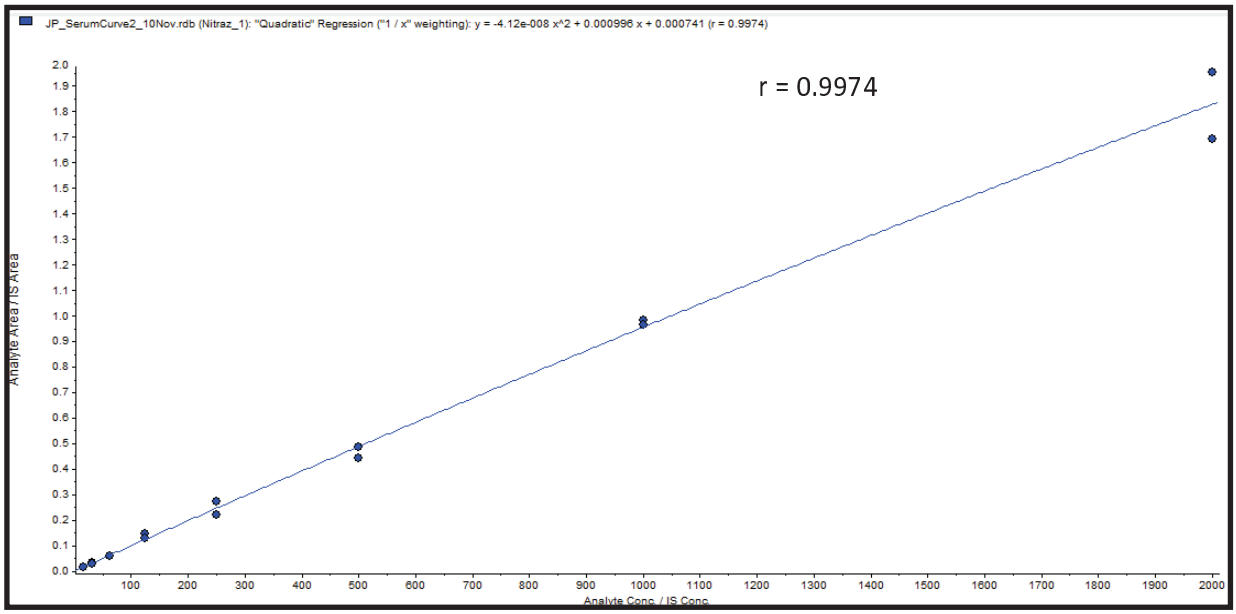


Figure B 26. Representative calibration curve for Nitrazepam: Validation 2, Day 2.

Table B 34.1. Nitrazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.3	0.24	1.6	98.4
31.3	S7	2 of 2	31.5	1.04	3.3	100.6
62.5	S6	2 of 2	58.5	0.17	0.3	93.6
125	S5	2 of 2	138	14.5	10.5	110.7
250	S4	2 of 2	249	38.6	15.5	99.7
500	S3	2 of 2	476	31.9	6.7	95.3
1000	S2	2 of 2	1020	14.9	1.5	102
2000	S1	2 of 2	2000	221	11.1	99.8

Table B 34.2. Summary of Nitrazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	15	0.18	1.2	96.4
45	L QC	2 of 2	50.2	0.32	0.6	111.6
800	M QC	2 of 2	814	1.74	0.2	101.8
1600	H QC	2 of 2	1600	115	7.2	99.8

Nitrazepam: Serum Validation 3, Day 2

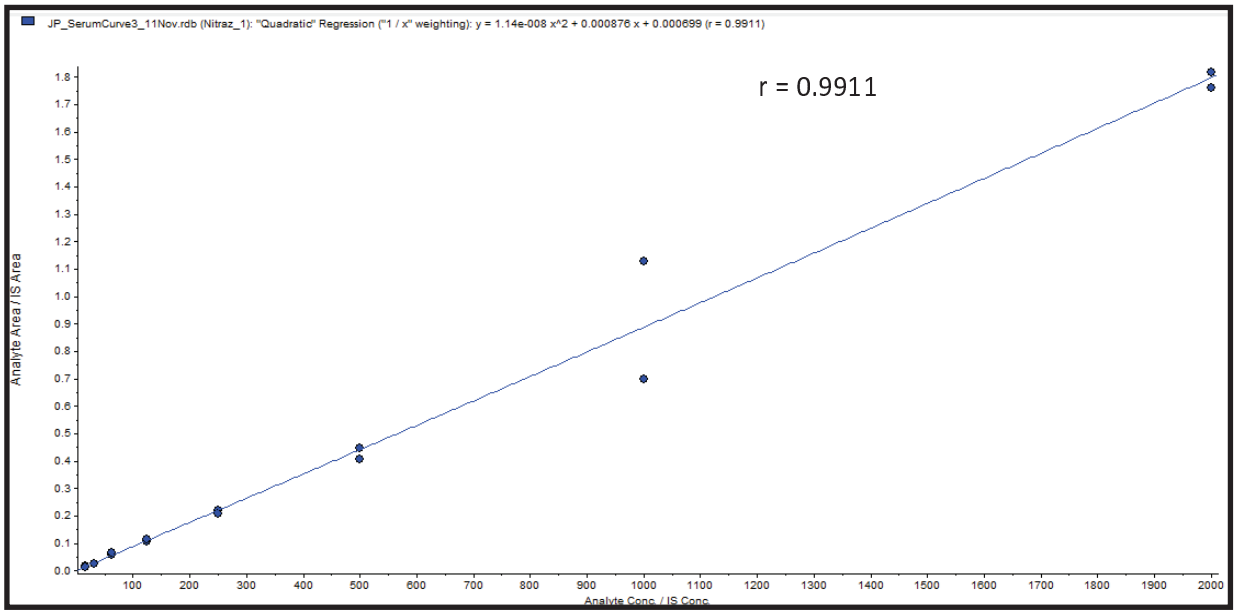


Figure B 27. Representative calibration curve for Nitrazepam: Validation 3, Day 2.

Table B 35.1. Nitrazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.4	2.66	17.3	98.7
31.3	S7	2 of 2	29.4	0.51	1.7	94
62.5	S6	2 of 2	69.3	4.24	6.1	110.9
125	S5	2 of 2	126	4.09	3.3	100.5
250	S4	2 of 2	242	8.85	3.7	96.7
500	S3	2 of 2	483	32.7	6.8	96.6
1000	S2	2 of 2	1030	337	32.8	102.9
2000	S1	2 of 2	1990	45.3	2.3	99.5

Table B 35.2. Summary of Nitrazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	14.8	3.23	21.8	94.9
45	L QC	2 of 2	41.9	9.48	22.6	93.1
800	M QC	2 of 2	838	0.65	0.1	104.8
1600	H QC	2 of 2	1860	93.4	5	116.2

Table B 36.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	14.6	1.13	7.5	93.9
31.3	S7	6 of 6	32	0.84	2.6	102.2
62.5	S6	6 of 6	62.1	2.25	3.5	99.3
125	S5	6 of 6	133	6.33	4.7	106.2
250	S4	6 of 6	246	23.7	9.6	98.2
500	S3	5 of 5	407	28	6.9	99
1000	S2	6 of 6	855	176	17.1	102.7
2000	S1	6 of 6	1650	99.1	5.5	98.1

Table B 36.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	14.9	1.63	11	95.5
45	L QC	6 of 6	49.7	4.24	9.5	110.5
800	M QC	6 of 6	871	1.35	0.2	108.9
1600	H QC	6 of 6	1890	137	7.1	117.9

Oxazepam: Serum Validation 1, Day1

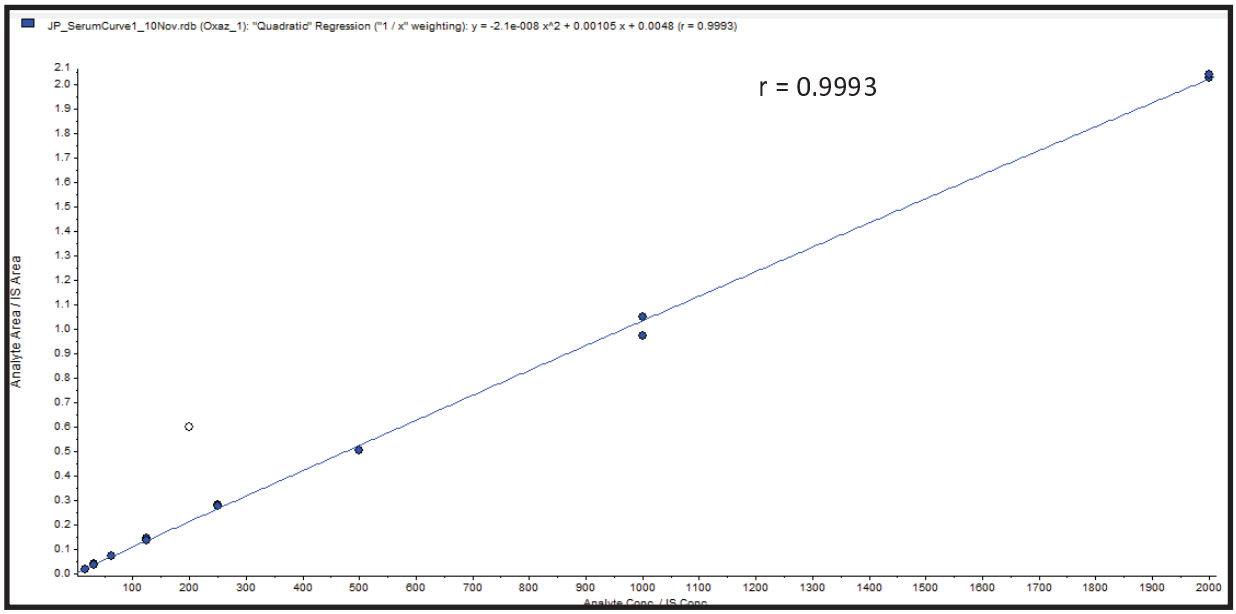


Figure B 28. Representative calibration curve for Oxazepam: Validation 1, Day 1.

Table B 37.1. Oxazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	12.8	0.17	1.4	82.3
31.3	S7	2 of 2	33.9	2.12	6.2	108.4
62.5	S6	2 of 2	65.1	0.52	0.8	104.1
125	S5	2 of 2	129	6.48	5	103.4
250	S4	2 of 2	263	0.72	0.3	105.2
500	S3	1 of 1	482	N/A	N/A	96.4
1000	S2	2 of 2	977	55.1	5.6	97.7
2000	S1	2 of 2	2010	10.9	0.5	100.6

Table B 27.2. Summary of Oxazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	19.7	8.71	44.2	126.2
45	L QC	2 of 2	53.4	0.35	0.7	118.7
800	M QC	2 of 2	999	93.7	9.4	124.9
1600	H QC	2 of 2	1850	7.88	0.4	115.4

Oxazepam: Serum Validation 2, Day 2

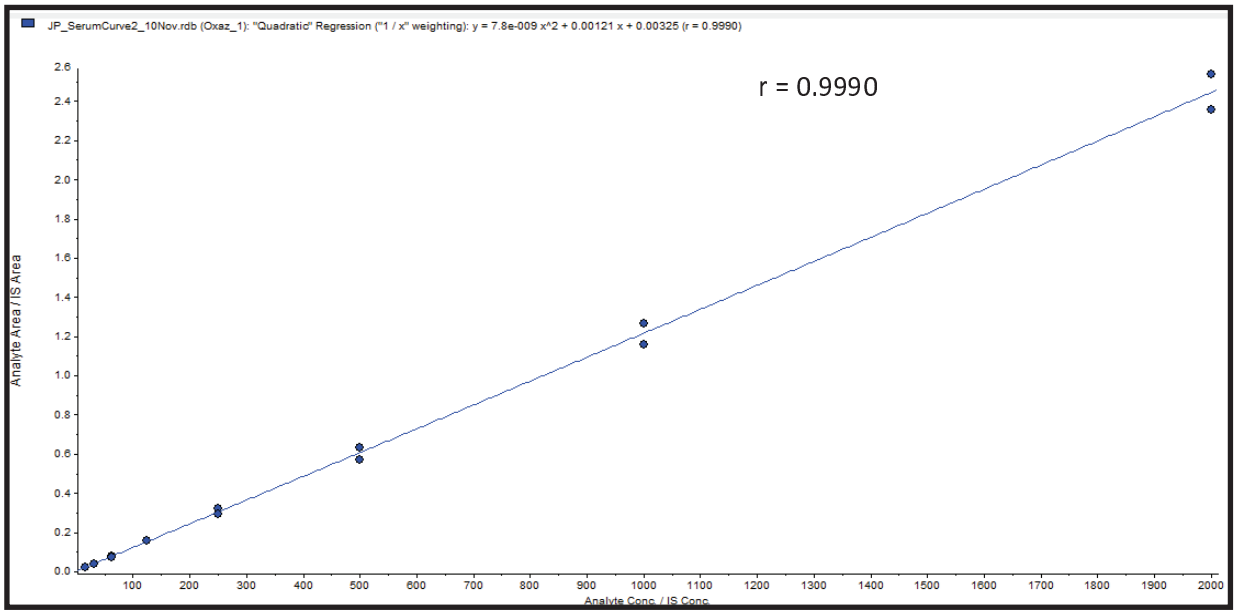


Figure B 29. Representative calibration curve for Oxazepam: Validation 2, Day 2.

Table B 38.1. Oxazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.2	0.72	4.4	103.6
31.3	S7	2 of 2	29.4	1.24	4.2	94
62.5	S6	2 of 2	61.4	3.5	5.7	98.2
125	S5	2 of 2	130	0.19	0.1	104.1
250	S4	2 of 2	254	16.5	6.5	101.5
500	S3	2 of 2	494	35.8	7.3	98.7
1000	S2	2 of 2	998	61.8	6.2	99.8
2000	S1	2 of 2	2000	104	5.2	100.1

Table B 38.2. Summary of Oxazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	14.9	1.59	10.6	95.8
45	L QC	2 of 2	42.1	0.44	1	93.5
800	M QC	2 of 2	804	4.85	0.6	100.6
1600	H QC	2 of 2	1450	190	13.1	90.8

Oxazepam: Serum Validation 3, Day 2

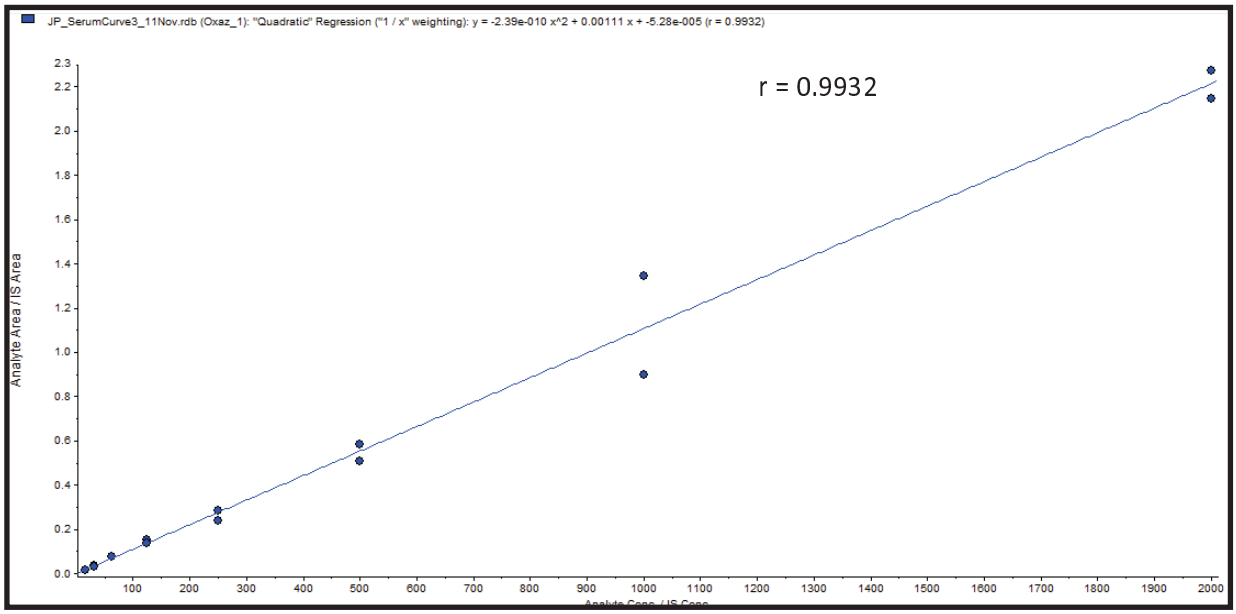


Figure B 30. Representative calibration curve for Oxazepam: Validation 3, Day 2.

Table B 39.1. Oxazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	14.6	0.73	5	93.4
31.3	S7	2 of 2	30.7	0.96	3.1	98.2
62.5	S6	2 of 2	68	0.34	0.5	108.9
125	S5	2 of 2	131	9.67	7.4	104.5
250	S4	2 of 2	237	29.2	12.3	95
500	S3	2 of 2	493	48.4	9.8	98.7
1000	S2	2 of 2	1010	283	27.9	101.4
2000	S1	2 of 2	2000	81.5	4.1	99.8

Table B39.2. Summary of Oxazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	16.7	2.45	14.7	106.8
45	L QC	2 of 2	43.5	3.67	8.4	96.6
800	M QC	2 of 2	737	20.1	2.7	92.1
1600	H QC	2 of 2	1740	62.5	3.6	108.5

Table B 40.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	14.5	0.54	3.6	93.1
31.3	S7	6 of 6	31.4	1.44	4.5	100.2
62.5	S6	6 of 6	64.8	1.45	2.3	103.7
125	S5	6 of 6	130	5.45	4.2	104
250	S4	6 of 6	246	22.9	9.4	98.2
500	S3	5 of 5	417	28.3	5.8	100.9
1000	S2	6 of 6	831	172	17.1	99.2
2000	S1	6 of 6	1660	80.2	5	99.2

Table B 40.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	17.1	4.25	23.2	109.6
45	L QC	6 of 6	46.3	1.49	3.4	102.9
800	M QC	6 of 6	847	39.6	4.2	105.8
1600	H QC	6 of 6	1680	86.7	5.7	104.9

<u>Temazepam</u>: Serum Validation 1, Day1

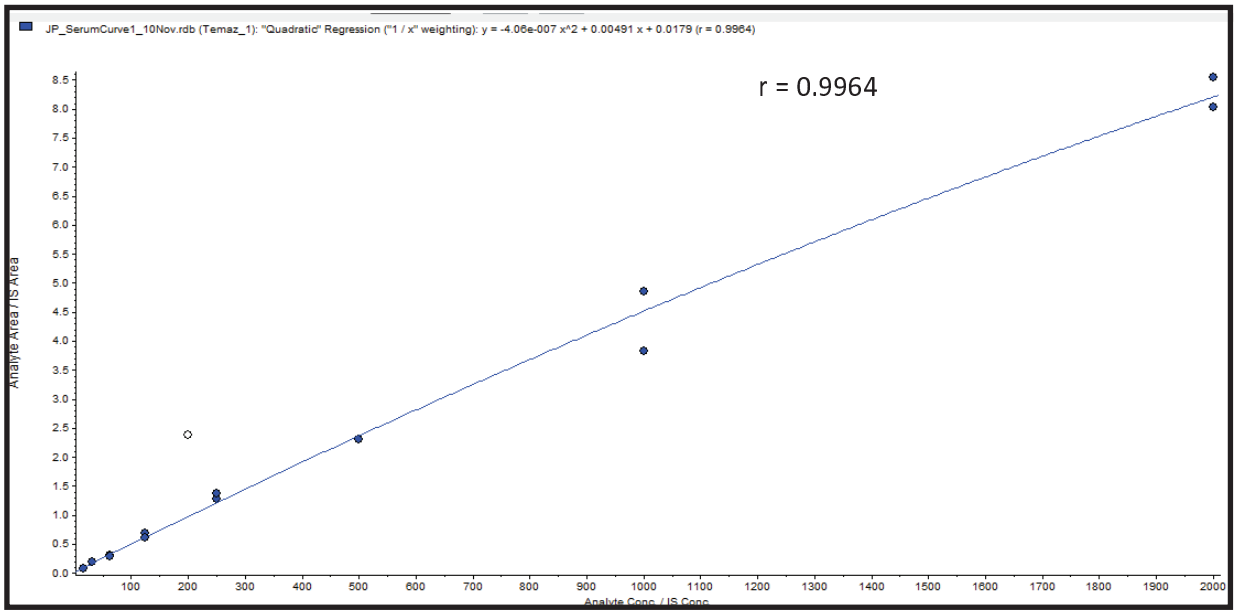


Figure B 31. Representative calibration curve for Temazepam: Validation 1, Day 1.

Table B 41.1. Temazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.3	0.64	4.8	85
31.3	S7	2 of 2	35.2	0.12	0.3	112.4
62.5	S6	2 of 2	58	1.13	1.9	92.9
125	S5	2 of 2	131	12.4	9.4	104.8
250	S4	2 of 2	273	15.1	5.5	109.4
500	S3	1 of 1	486	N/A	N/A	97.2
1000	S2	2 of 2	960	175	18.3	96
2000	S1	2 of 2	2020	113	5.6	101.2

Table B 41.2. Summary of Temazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	15.2	0.89	5.9	97.6
45	L QC	2 of 2	49.5	4.13	8.3	110
800	M QC	2 of 2	896	39.4	4.4	112.1
1600	H QC	2 of 2	1890	171	9.1	118.4

Temazepam: Serum Validation 2, Day 2

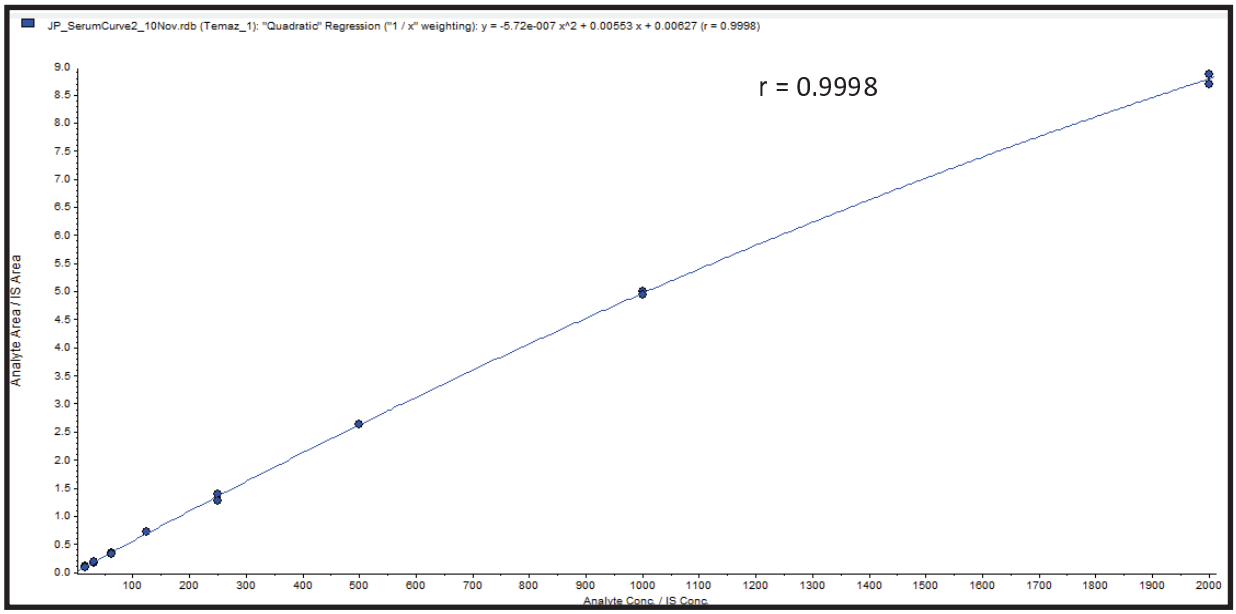


Figure B 32. Representative calibration curve for Temazepam: Validation 2, Day 2.

Table B 42.1. Temazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16	1.91	11.9	102.8
31.3	S7	2 of 2	30.2	3.38	11.2	96.4
62.5	S6	2 of 2	61.4	1.96	3.2	98.2
125	S5	2 of 2	130	0.05	0	104.2
250	S4	2 of 2	246	14.8	6	98.3
500	S3	2 of 2	500	0.51	0.1	100
1000	S2	2 of 2	1000	10.8	1.1	100.1
2000	S1	2 of 2	2000	37.6	1.9	100

Table B 42.2. Summary of Temazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	14.9	1.28	8.6	95.7
45	L QC	2 of 2	47	2.18	4.6	104.4
800	M QC	2 of 2	770	90	11.7	96.2
1600	H QC	2 of 2	1610	109	6.7	100.9

Temazepam: Serum Validation 3, Day 2

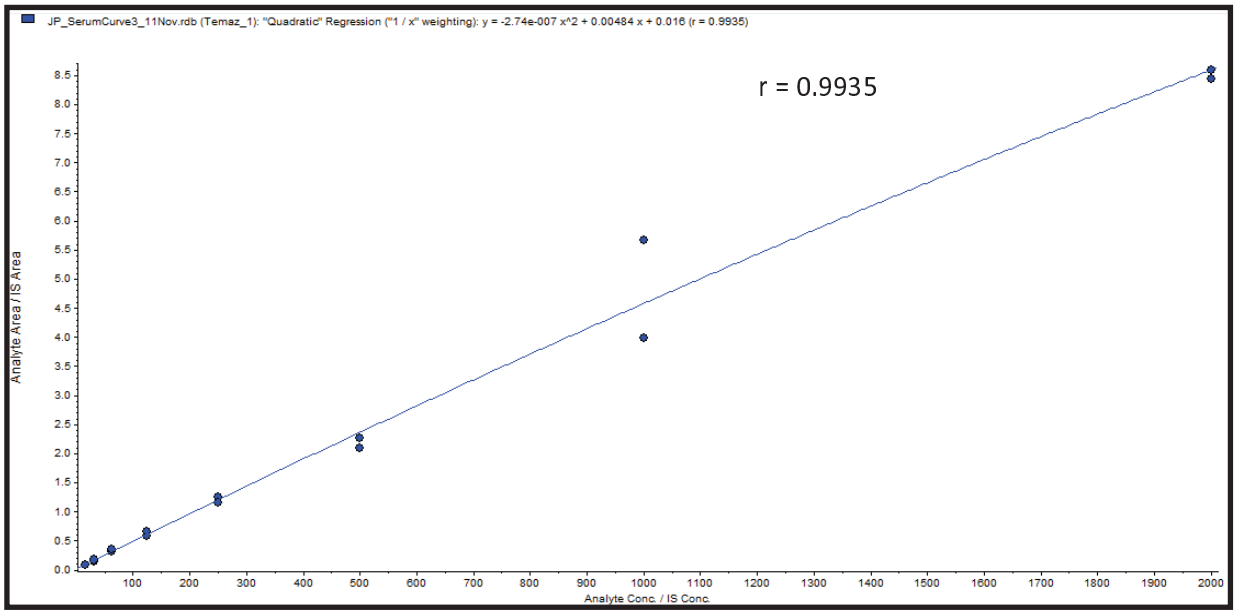


Figure B 33. Representative calibration curve for Temazepam: Validation 3, Day 2.

Table B 43.1. Temazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.5	1.6	10.3	99.2
31.3	S7	2 of 2	30.2	3.51	11.6	96.4
62.5	S6	2 of 2	67.1	4.79	7.1	107.4
125	S5	2 of 2	125	9.85	7.8	100.4
250	S4	2 of 2	250	14.9	6	99.9
500	S3	2 of 2	460	24.5	5.3	91.9
1000	S2	2 of 2	1060	281	26.5	105.9
2000	S1	2 of 2	1980	27.5	1.4	99

Table B 43.2. Summary of Temazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	12.6	0.78	6.2	80.7
45	L QC	2 of 2	42.5	3.41	8	94.5
800	M QC	2 of 2	766	26.7	3.5	95.8
1600	H QC	2 of 2	1740	56.9	3.3	109

Table B44.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	14.9	1.38	9	95.7
31.3	S7	6 of 6	31.8	2.34	7.7	101.7
62.5	S6	6 of 6	62.2	2.63	4.1	99.5
125	S5	6 of 6	129	7.42	5.8	103.1
250	S4	6 of 6	248	14.9	6	99.1
500	S3	5 of 5	411	13.4	3.6	100.4
1000	S2	6 of 6	849	146	13.8	101.1
2000	S1	6 of 6	1650	80.1	7.2	98.3

Table B 44.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	14.2	0.99	6.9	91.3
45	L QC	6 of 6	46.3	3.24	7	103
800	M QC	6 of 6	811	52	6.5	101.4
1600	H QC	6 of 6	1750	112	6.3	109.4

Here follows a list of %Accuracy values that did not fall within the acceptance criteria values for calibration standards and quality controls:

Table B 45%Accuracy values that did not fit acceptance criteria and an indication of the amount of percent with which it was too high or too low to get to either the lowest or highest accepted percentage.

Analyte	Validation Batch	STD/QC Fail	%Accuracy	+/- % out*
Nortriptyline	Validation 1	H QC	131.8587	- 17
Amitriptyline	Validation 1	H QC	128.061	- 13
Alprazolam	Validation 1	S7	117.7782	- 3
		H QC	119.3722	- 4
	Validation 3	LLOQ	72.71141	+ 12
Clonazepam	Validation 1	M QC	123.09	- 7
		H QC	129.1529	- 14
Lorazepam	Validation 1	L QC	120.098	- 5
		M QC	116.0236	- 1
		H QC	121.668	- 6
Nitrazepam	Validation 1	L QC	126.7986	- 12
		M QC	120.1817	- 5
		H QC	137.7703	- 22
	Validation 3	H QC	116.1938	- 1
	Overall	H QC	117.9348	- 3
Oxazepam	Validation 1	LLOQ	126.229	- 11
		L QC	118.665	- 4
		M QC	124.8574	- 10
Temazepam	Validation 1	H QC	118.3845	- 3

*Rounding up to get to lowest or highest accepted %Accuracy values

C: Urine Accuracy and Precision Validation Results

Here follows a list for the series of results that is presented for the Urine Accuracy and Precision Results. It can be seen that the following sequence of results is presented for each analyte:

- A representative calibration curve (including r^2 values)
- Calibration standard accuracy and precision results
- Summary of intra-validation quality control standards

} Presented for validation 1, 2 and 3 for each analyte

- Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3
- Overall Quality Control Accuracy and Precision Estimation

Nortriptyline: Urine Validation 1, Day1

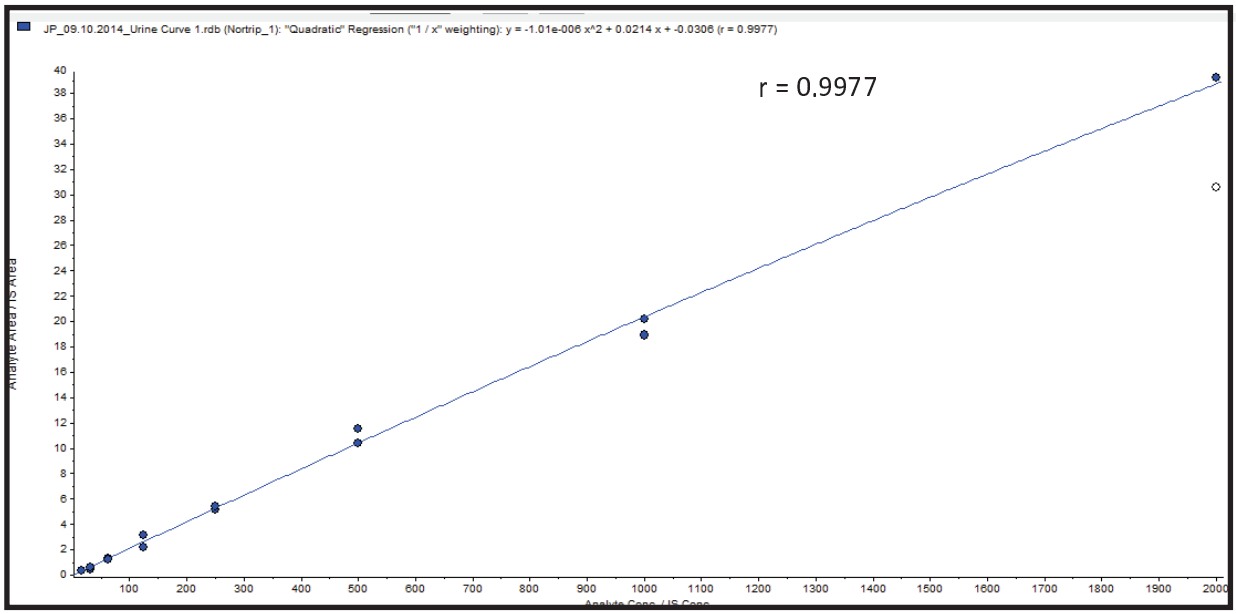


Figure C 1. Representative calibration curve for Nortriptyline: Validation 1, Day 1.

Table C 1.1. Nortriptyline Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	17.7	0.53	3	113.7
31.3	S7	2 of 2	26	4.3	16.5	83.2
62.5	S6	2 of 2	61.9	2.33	3.8	99.1
125	S5	2 of 2	127	29.6	23.3	101.4
250	S4	2 of 2	251	9.52	3.8	100.5
500	S3	2 of 2	528	40.7	7.7	105.5
1000	S2	2 of 2	959	46.7	4.9	95.9
2000	S1	1 of 2	2030	N/A	N/A	101.5

Table C 1.2. Summary of Nortriptyline intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	12.1	2.25	18.7	77.4
45	L QC	2 of 2	51.2	8.15	15.9	113.7
800	M QC	2 of 2	713	123	17.2	89.1
1600	H QC	2 of 2	1980	621	31.5	123.5

Nortriptyline: Urine Validation 2, Day 2

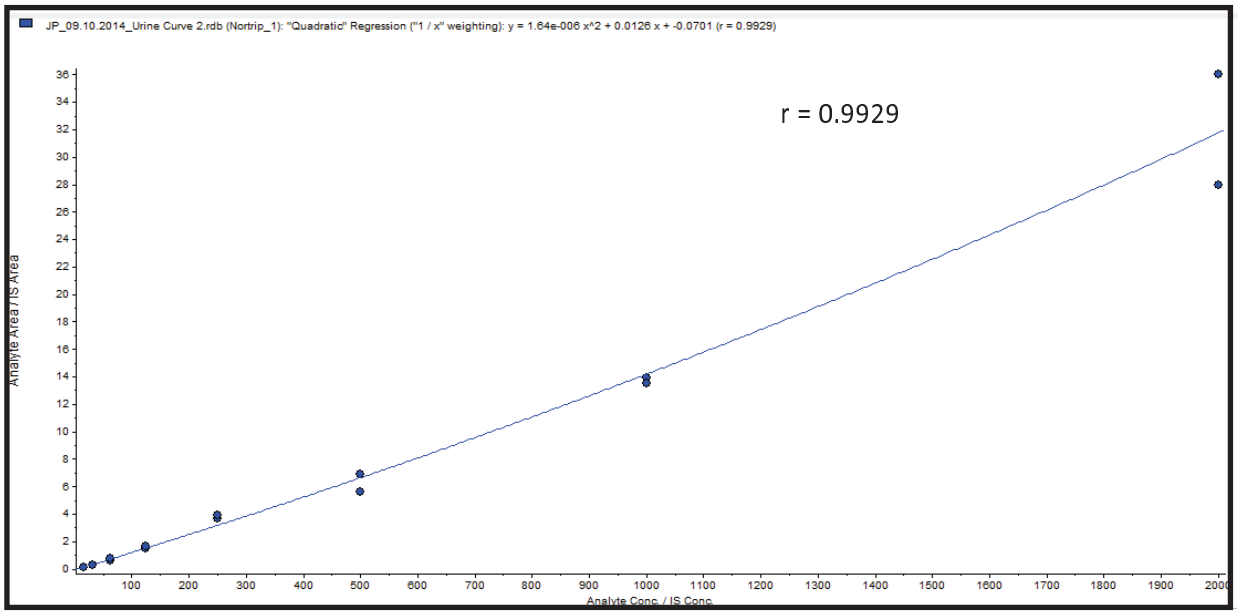


Figure C 2. Representative calibration curve for Nortriptyline: Validation 2, Day 2.

Table C 2.1. Nortriptyline Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.6	0.54	3.4	100.3
31.3	S7	2 of 2	27.7	0.31	1.1	88.6
62.5	S6	2 of 2	60.6	9.31	15.4	96.9
125	S5	2 of 2	129	9.79	7.6	103.6
250	S4	2 of 2	295	13.3	4.5	118.1
500	S3	2 of 2	472	62	13.2	94.3
1000	S2	2 of 2	972	17.6	1.8	97.2
2000	S1	2 of 2	2010	298	14.8	100.4

Table C 2.2. Summary of Nortriptyline intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	15	3.88	26	95.9
45	L QC	2 of 2	55.3	5.46	9.9	122.9
800	M QC	2 of 2	937	126	13.5	117.2
1600	H QC	2 of 2	1830	342	18.7	114.5

Nortriptyline: Urine Validation 3, Day 3

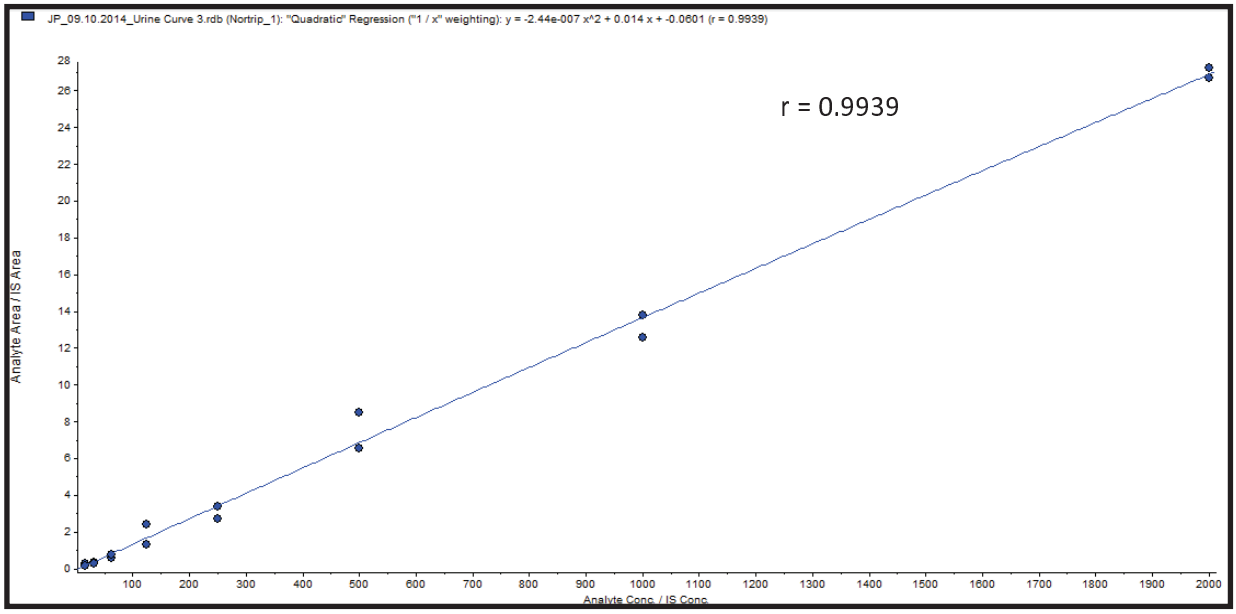


Figure C 3. Representative calibration curve for Nortriptyline: Validation 3, Day 3.

Table C 3.1. Nortriptyline Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	18.8	5.17	27.5	120.7
31.3	S7	2 of 2	27.2	2.13	7.9	86.8
62.5	S6	2 of 2	53.5	8.51	15.9	85.6
125	S5	2 of 2	138	57	41.5	110.1
250	S4	2 of 2	225	34.2	15.2	89.9
500	S3	2 of 2	549	101	18.5	109.9
1000	S2	2 of 2	966	62.5	6.5	96.6
2000	S1	2 of 2	2010	28.8	1.4	100.4

Table C 3.2. Summary of Nortriptyline intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	18.3	3.87	21.2	117.1
45	L QC	2 of 2	52.3	0.31	0.6	116.1
800	M QC	2 of 2	745	24	3.2	93.1
1600	H QC	2 of 2	1780	179	10	111.5

Table C 4.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	17.4	2.08	11.3	111.6
31.3	S7	6 of 6	27	2.25	8.5	86.2
62.5	S6	6 of 6	58.7	6.72	11.7	93.9
125	S5	6 of 6	131	32.1	24.1	105
250	S4	6 of 6	257	19	7.8	102.8
500	S3	6 of 6	516	68	13.1	103.2
1000	S2	6 of 6	966	42.3	4.4	96.6
2000	S1	5 of 6	2020	163	8.1	100.8

Table C 4.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	15.1	3.34	21.9	96.8
45	L QC	6 of 6	52.9	4.64	8.8	117.6
800	M QC	6 of 6	798	91.1	11.3	99.8
1600	H QC	6 of 6	1860	381	20.1	116.5

Amitriptyline: Urine Validation 1, Day1

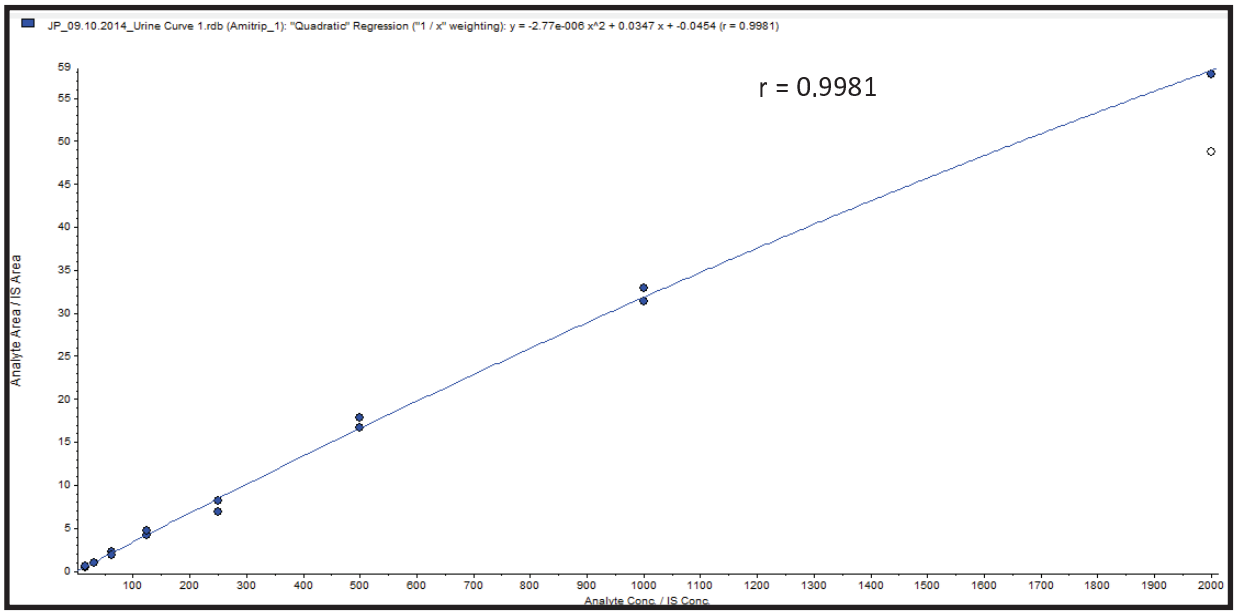


Figure C 4. Representative calibration curve for Amitriptyline: Validation 1, Day 1.

Table C 5.1. Amitriptyline Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.5	3.29	19.9	105.7
31.3	S7	2 of 2	30.2	0.5	1.6	96.4
62.5	S6	2 of 2	61.8	6.55	10.6	98.8
125	S5	2 of 2	132	9.18	7	105.6
250	S4	2 of 2	222	26.6	12	88.6
500	S3	2 of 2	520	27.5	5.3	104.1
1000	S2	2 of 2	1010	38.9	3.8	101
2000	S1	1 of 2	1980	N/A	N/A	99.1

Table C 5.2. Summary of Amitriptyline intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	11	0.58	5.2	70.7
45	L QC	2 of 2	53.6	2.39	4.5	119
800	M QC	2 of 2	751	119	15.8	93.9
1600	H QC	2 of 2	2220	708	31.9	138.9

Amitriptyline: Urine Validation 2, Day 2

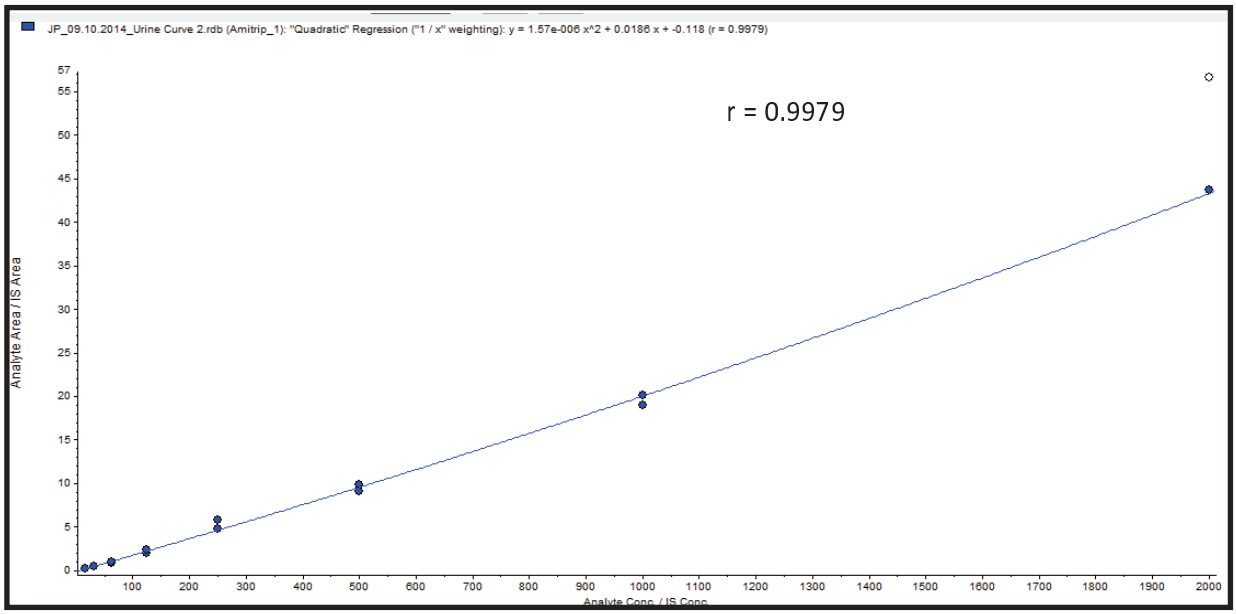


Figure C 5. Representative calibration curve for Amitriptyline: Validation 2, Day 2.

Table C 6.1. Amitriptyline Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	17.5	0.06	0.3	112.5
31.3	S7	2 of 2	29.9	0.52	1.7	95.5
62.5	S6	2 of 2	53.3	4.62	8.7	85.3
125	S5	2 of 2	120	14	11.6	96.3
250	S4	2 of 2	282	37	13.1	112.9
500	S3	2 of 2	495	24.3	4.9	98.9
1000	S2	2 of 2	979	37.6	3.8	97.9
2000	S1	1 of 2	2020	N/A	N/A	100.8

Table C 6.2. Summary of Amitriptyline intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	18.4	2.9	15.8	117.6
45	L QC	2 of 2	52.8	1.48	2.8	117.4
800	M QC	2 of 2	925	151	16.4	115.6
1600	H QC	2 of 2	1990	312	15.7	124.1

Amitriptyline: Urine Validation 3, Day 3

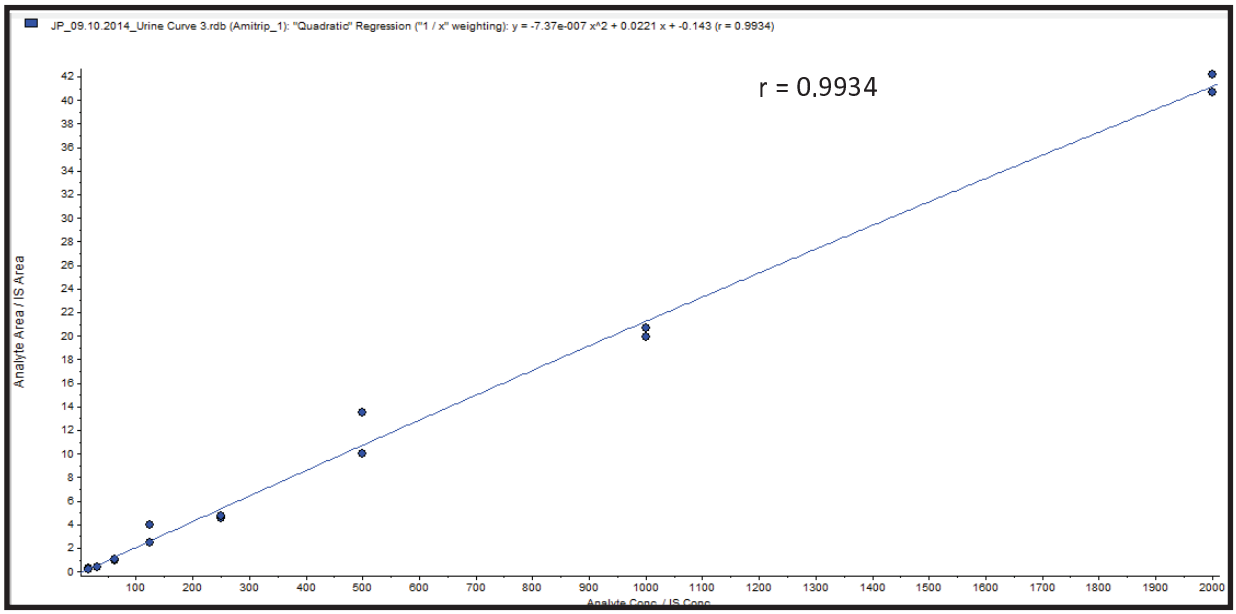


Figure C 6. Representative calibration curve for Amitriptyline: Validation 3, Day 3.

Table C 7.1. Amitriptyline Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	18.4	4.35	23.6	118
31.3	S7	2 of 2	25.6	0.47	1.9	81.7
62.5	S6	2 of 2	52.9	4.93	9.3	84.7
125	S5	2 of 2	153	46.1	30.1	122.7
250	S4	2 of 2	218	5.55	2.5	87.3
500	S3	2 of 2	547	117	21.3	109.5
1000	S2	2 of 2	955	24	2.5	95.5
2000	S1	2 of 2	2010	56	2.8	100.7

Table C 7.2. Summary of Amitriptyline intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	19.8	3.69	18.7	126.6
45	L QC	2 of 2	54.3	0.95	1.8	120.7
800	M QC	2 of 2	742	34.8	4.7	92.7
1600	H QC	2 of 2	1720	224	13.1	107.4

Table C 8.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	17.5	2.56	14.6	112.1
31.3	S7	6 of 6	28.5	0.5	1.7	91.2
62.5	S6	6 of 6	56	5.37	9.5	89.6
125	S5	6 of 6	135	23.1	16.2	108.2
250	S4	6 of 6	241	23	9.2	96.3
500	S3	6 of 6	521	56.2	10.5	104.2
1000	S2	6 of 6	981	33.5	3.4	98.1
2000	S1	4 of 6	2000	56	2.8	100.2

Table C 8.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	16.4	2.39	13.2	105
45	L QC	6 of 6	53.6	1.61	3	119
800	M QC	6 of 6	806	102	12.3	100.7
1600	H QC	6 of 6	1980	415	20.2	123.5

Citalopram: Urine Validation 1, Day 1

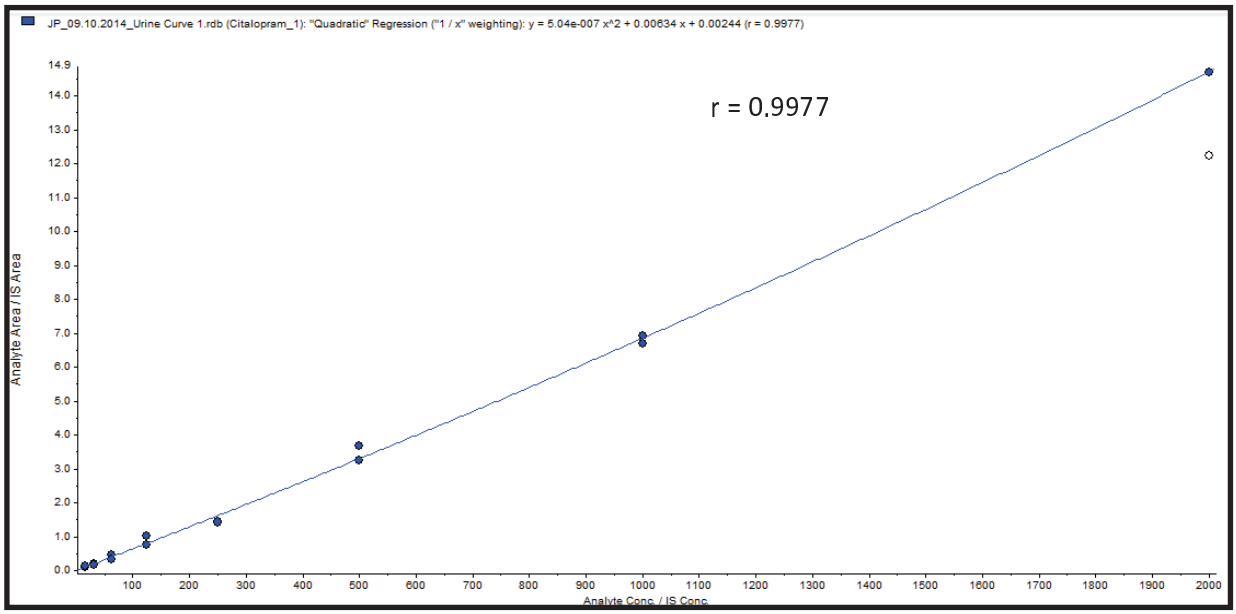


Figure C 7. Representative calibration curve for Citalopram: Validation 1, Day 1.

Table C 9.1. Citalopram Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.6	2.31	13.9	106.7
31.3	S7	2 of 2	28.6	0.95	3.3	91.5
62.5	S6	2 of 2	61.5	13	21.2	98.3
125	S5	2 of 2	138	29.4	21.3	110.7
250	S4	2 of 2	221	0.41	0.2	88.4
500	S3	2 of 2	523	43.5	8.3	104.6
1000	S2	2 of 2	995	22.9	2.3	99.5
2000	S1	1 of 2	2000	N/A	N/A	100

Table C 9.2. Summary of Citalopram intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	12.9	4.73	36.6	82.9
45	L QC	2 of 2	49.4	4.46	9	109.9
800	M QC	2 of 2	709	69.5	9.8	88.6
1600	H QC	2 of 2	1960	356	18.1	122.6

Citalopram: Urine Validation 2, Day 2

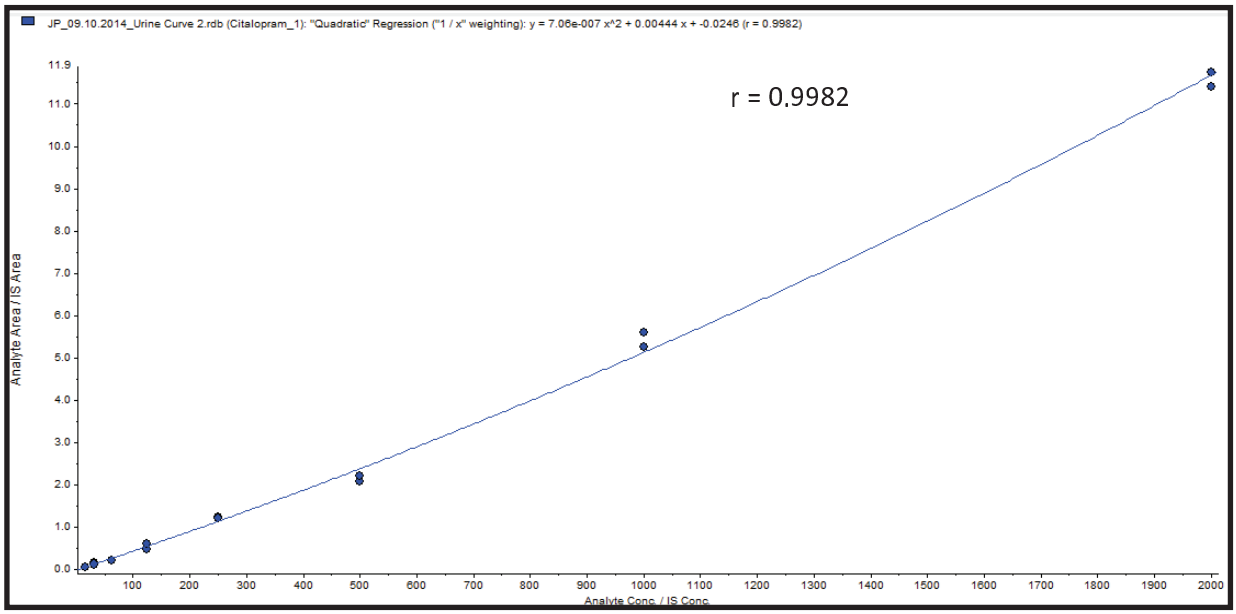


Figure C 8. Representative calibration curve for Citalopram: Validation 2, Day 2.

Table C 10.1. Citalopram Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	17.1	2.33	13.7	109.5
31.3	S7	2 of 2	33.9	4.35	12.8	108.5
62.5	S6	2 of 2	51.6	3.15	6.1	82.6
125	S5	2 of 2	121	21	17.3	97.1
250	S4	2 of 2	267	3.34	1.2	106.9
500	S3	2 of 2	453	20.2	4.5	90.7
1000	S2	2 of 2	1050	38.8	3.7	105.1
2000	S1	2 of 2	1990	32.5	1.6	99.3

Table C 10.2. Summary of Citalopram intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	**Sample Name**	**n**	**Mean**	**Standard Deviation**	**%CV**	**%Accuracy**
15.6	LLOQ	2 of 2	17.1	1.2	7	109.7
45	L QC	2 of 2	48.5	1.54	3.2	107.8
800	M QC	2 of 2	906	246	27.2	113.2
1600	H QC	2 of 2	1820	280	15.4	113.5

Citalopram: Urine Validation 3, Day 3

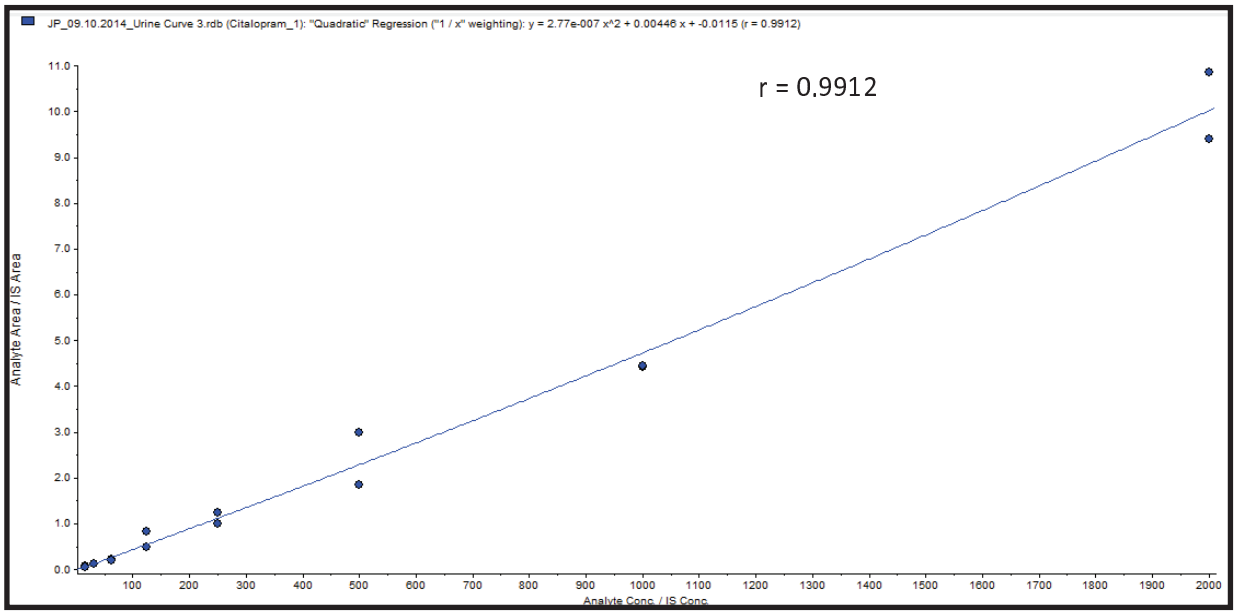


Figure C 9. Representative calibration curve for Citalopram: Validation 3, Day 3.

Table C 11.1. Citalopram Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	17.1	4.43	25.9	109.4
31.3	S7	2 of 2	28.5	0.34	1.2	91.1
62.5	S6	2 of 2	50.1	1.2	2.4	80.1
125	S5	2 of 2	147	52.6	35.8	117.6
250	S4	2 of 2	251	36.7	14.6	100.5
500	S3	2 of 2	528	169	31.9	105.7
1000	S2	2 of 2	943	2.1	0.2	94.3
2000	S1	2 of 2	2020	186	9.2	100.9

Table C 11.2. Summary of Citalopram intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	17.7	3.41	19.3	113.4
45	L QC	2 of 2	51.3	10.9	21.2	114
800	M QC	2 of 2	809	185	22.9	101.1
1600	H QC	2 of 2	1950	277	14.2	121.8

Table C 12.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	16.9	3.02	17.8	108.5
31.3	S7	6 of 6	30.4	1.88	5.8	97
62.5	S6	6 of 6	54.4	5.79	9.9	87
125	S5	6 of 6	136	34.4	24.8	108.5
250	S4	6 of 6	247	13.5	5.3	98.6
500	S3	6 of 6	502	77.4	14.9	100.3
1000	S2	6 of 6	996	21.3	2.1	99.6
2000	S1	5 of 6	2000	109	5.4	100.1

Table C 12.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	15.9	3.12	21	102
45	L QC	6 of 6	49.7	5.62	11.1	110.5
800	M QC	6 of 6	808	167	20	101
1600	H QC	6 of 6	1910	304	15.9	119.3

Alprazolam: Urine Validation 1, Day1

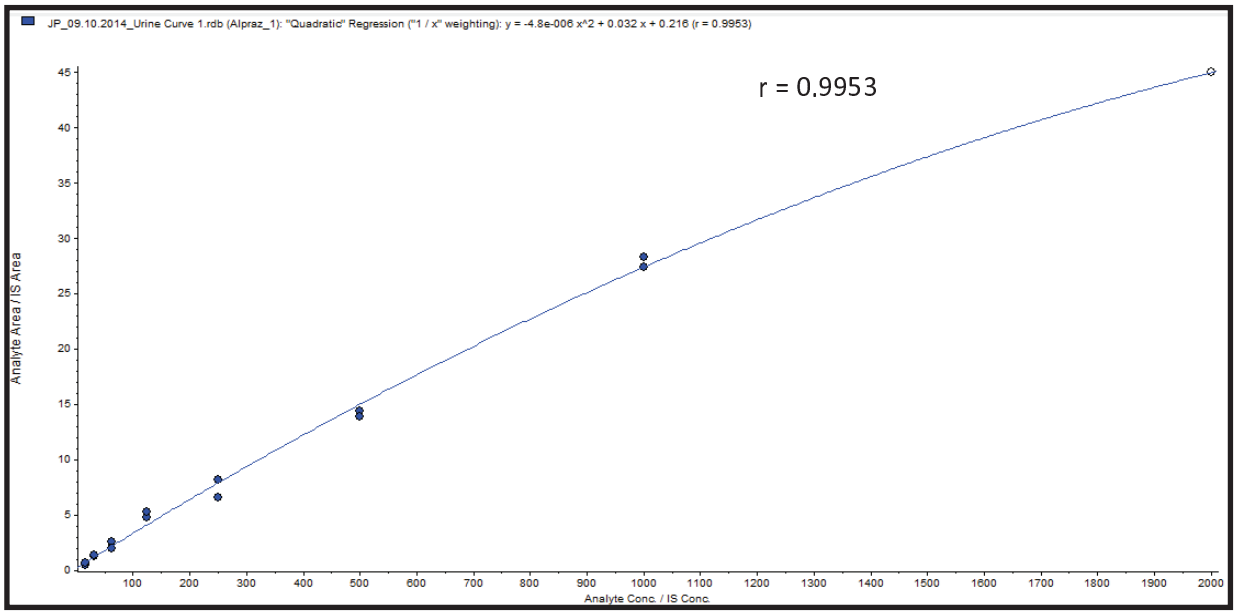


Figure C 10. Representative calibration curve for Alprazolam: Validation 1, Day 1.

Table C 13.1. Alprazolam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	10.9	3.96	36.3	69.9
31.3	S7	2 of 2	35.6	0.75	2.1	113.8
62.5	S6	2 of 2	65.3	11.7	17.9	104.5
125	S5	2 of 2	155	12.3	8	123.8
250	S4	2 of 2	232	36.8	15.9	92.8
500	S3	2 of 2	467	13	2.8	93.4
1000	S2	2 of 2	1020	28.4	2.8	102
2000	S1	1 of 2	2000	N/A	N/A	100.1

Table C 13.2. Summary of Alprazolam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	8.6	2.43	28.3	55.1
45	L QC	2 of 2	36.1	5.98	16.5	80.3
800	M QC	2 of 2	721	29.5	4.1	90.1
1600	H QC	1 of 2	1850	N/A	N/A	115.7

Alprazolam: Urine Validation 2, Day 2

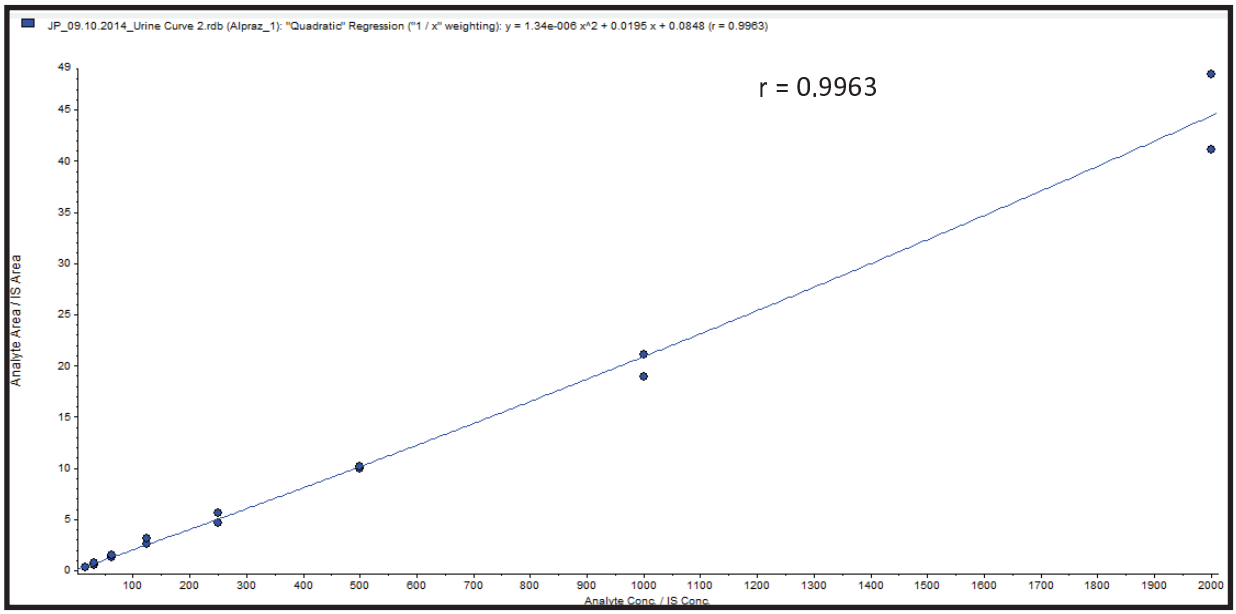


Figure C 11. Representative calibration curve for Alprazolam: Validation 2, Day 2.

Table C 14.1. Alprazolam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	12.4	0.39	3.1	79.5
31.3	S7	2 of 2	31.2	7.25	23.2	99.8
62.5	S6	2 of 2	67.4	7.26	10.8	107.9
125	S5	2 of 2	141	17.7	12.5	113.2
250	S4	2 of 2	257	34	13.2	102.9
500	S3	2 of 2	497	6.04	1.2	99.5
1000	S2	2 of 2	961	68.6	7.1	96.1
2000	S1	2 of 2	2010	209	10.4	100.7

Table C 14.2. Summary of Alprazolam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	13	2.34	18	83.5
45	L QC	2 of 2	49.8	3.03	6.1	110.7
800	M QC	2 of 2	903	154	17	112.9
1600	H QC	2 of 2	1750	234	13.3	109.6

Alprazolam: Urine Validation 3, Day 3

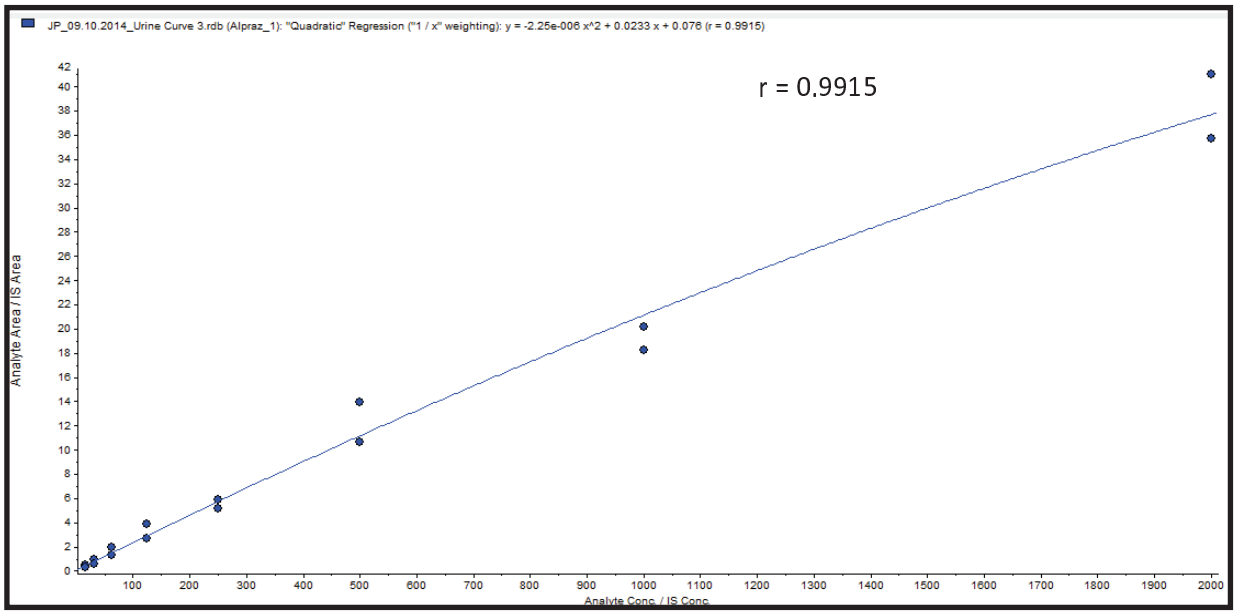


Figure C 12. Representative calibration curve for Alprazolam: Validation 3, Day 3.

Table C 15.1. Alprazolam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.5	0.52	3.1	105.9
31.3	S7	2 of 2	28.5	2.33	8.2	91.1
62.5	S6	2 of 2	65.7	12.8	19.5	105.2
125	S5	2 of 2	112	34.4	30.7	89.6
250	S4	2 of 2	268	36.5	13.6	107.3
500	S3	2 of 2	530	170	32	106
1000	S2	2 of 2	941	103	10.9	94.1
2000	S1	2 of 2	2030	8.68	0.4	101.6

Table C 15.2. Summary of Alprazolam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	16.5	0.15	0.9	105.7
45	L QC	2 of 2	62.3	8.92	14.3	138.3
800	M QC	2 of 2	828	71.3	8.6	103.6
1600	H QC	2 of 2	1870	369	19.8	116.6

Table C 16.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	13.3	1.62	14.2	85.1
31.3	S7	6 of 6	31.8	3.45	11.2	101.6
62.5	S6	6 of 6	66.2	10.6	16.1	105.9
125	S5	6 of 6	136	21.5	17.1	108.8
250	S4	6 of 6	252	35.8	14.2	101
500	S3	6 of 6	498	62.9	12	99.6
1000	S2	6 of 6	974	66.6	6.9	97.4
2000	S1	5 of 6	2020	109	5.4	100.8

Table C 16.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	12.7	1.64	15.7	81.4
45	L QC	6 of 6	49.4	5.98	12.3	109.8
800	M QC	6 of 6	817	84.9	9.9	102.2
1600	H QC	5 of 6	1820	302	16.6	114

Clonazepam: Urine Validation 1, Day1

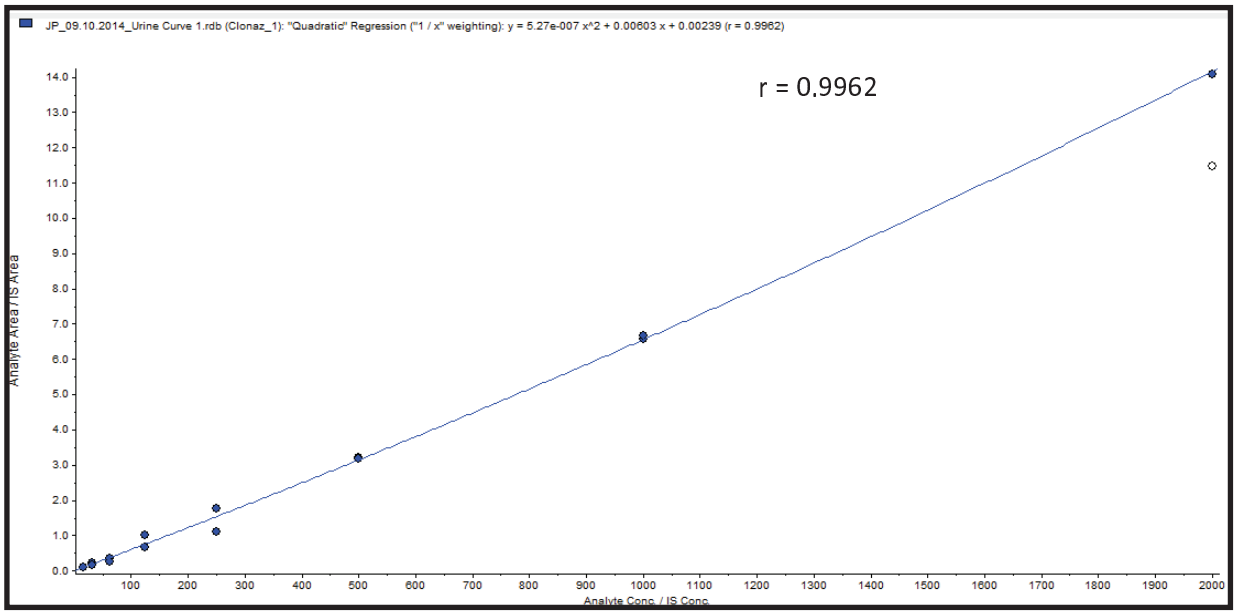


Figure C 13. Representative calibration curve for Clonazepam: Validation 1, Day 1.

Table C 17.1. Clonazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	17.5	1.98	11.4	111.9
31.3	S7	2 of 2	32.1	5.43	16.9	102.5
62.5	S6	2 of 2	49.6	10.4	20.9	79.3
125	S5	2 of 2	138	37	26.9	110
250	S4	2 of 2	233	71.1	30.5	93.4
500	S3	2 of 2	509	4.73	0.9	101.7
1000	S2	2 of 2	1010	10.2	1	101
2000	S1	1 of 2	1990	N/A	N/A	99.5

Table C 17.2. Summary of Clonazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	12.1	1.28	10.6	77.4
45	L QC	2 of 2	45.6	3.51	7.7	101.3
800	M QC	2 of 2	756	83.3	11	94.5
1600	H QC	2 of 2	2070	449	21.7	129.2

Clonazepam: Urine Validation 2, Day 2

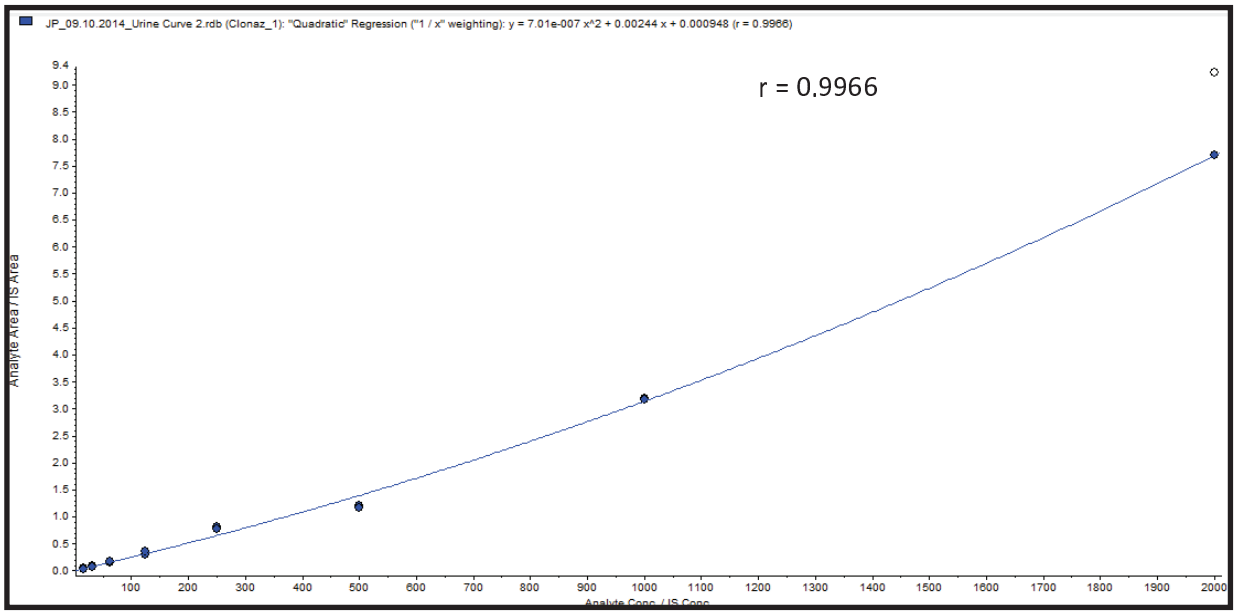


Figure C 14. Representative calibration curve for Clonazepam: Validation 2, Day 2.

Table C 18.1. Clonazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.4	3.87	28.9	85.7
31.3	S7	2 of 2	30.5	4.78	15.7	97.4
62.5	S6	2 of 2	65.7	10.3	15.7	105.1
125	S5	2 of 2	128	18.6	14.5	102.7
250	S4	2 of 2	301	8.64	2.9	120.4
500	S3	2 of 2	434	8.63	2	86.7
1000	S2	2 of 2	1010	5.17	0.5	101
2000	S1	1 of 2	2010	N/A	N/A	100.3

Table C 18.2. Summary of Clonazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	17.9	4.05	22.7	114.6
45	L QC	2 of 2	37.6	3.45	9.2	83.6
800	M QC	2 of 2	965	241	25	120.6
1600	H QC	2 of 2	1790	230	12.9	111.9

Clonazepam: Urine Validation 3, Day 3

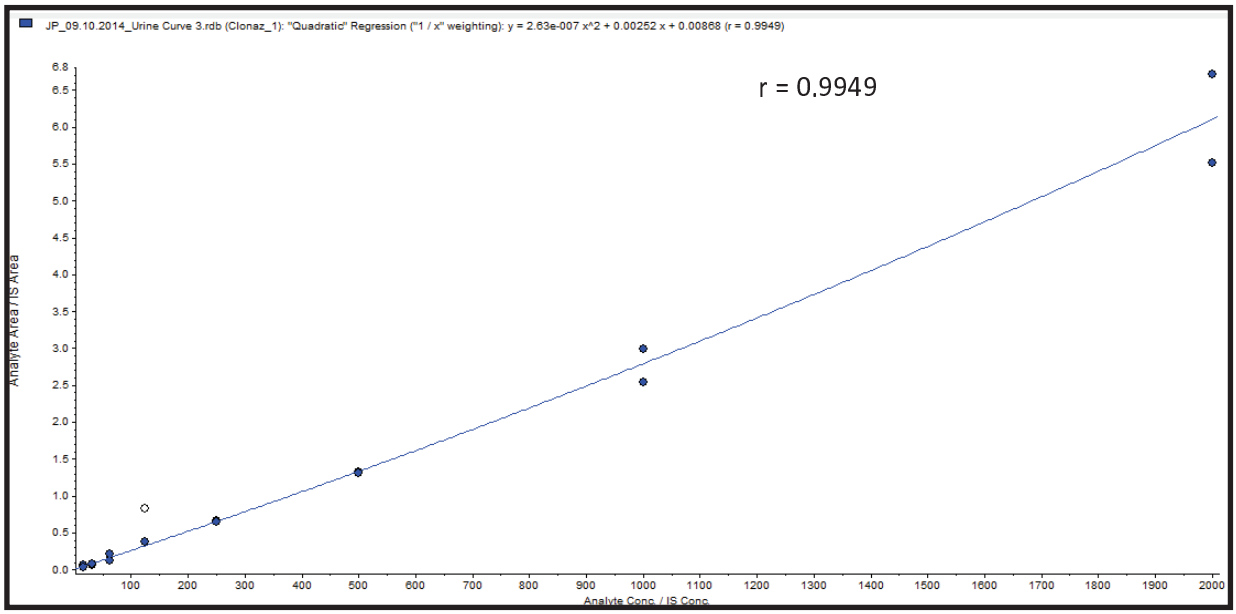


Figure C 15. Representative calibration curve for Clonazepam: Validation 3, Day 3.

Table C 19.1. Clonazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.5	9.33	56.6	105.7
31.3	S7	2 of 2	26.6	2	7.5	85
62.5	S6	2 of 2	64.1	27.5	42.9	102.6
125	S5	1 of 2	144	N/A	N/A	114.9
250	S4	2 of 2	251	6.15	2.4	100.4
500	S3	2 of 2	496	2.77	0.6	99.1
1000	S2	2 of 2	992	103	10.4	99.2
2000	S1	2 of 2	2000	237	11.9	100.1

Table C 19.2. Summary of Clonazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	10.7	6.46	60.4	68.6
45	L QC	2 of 2	53.4	13.7	25.7	118.6
800	M QC	2 of 2	802	14.2	1.8	100.3
1600	H QC	2 of 2	1940	183	9.4	121.4

Table C 20.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	15.8	5.06	32.3	101.1
31.3	S7	6 of 6	29.7	4.07	13.4	95
62.5	S6	6 of 6	59.8	16.1	26.5	95.7
125	S5	5 of 6	136	27.8	20.7	109.2
250	S4	6 of 6	262	28.6	11.9	104.7
500	S3	6 of 6	479	5.38	1.2	95.9
1000	S2	6 of 6	1000	39.5	4	100.4
2000	S1	4 of 6	2000	237	11.9	99.9

Table C 20.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	13.6	3.93	31.2	86.9
45	L QC	6 of 6	45.5	6.89	14.2	101.2
800	M QC	6 of 6	841	113	12.6	105.1
1600	H QC	6 of 6	1930	287	14.7	120.8

<u>Diazepam</u>: Urine Validation 1, Day1

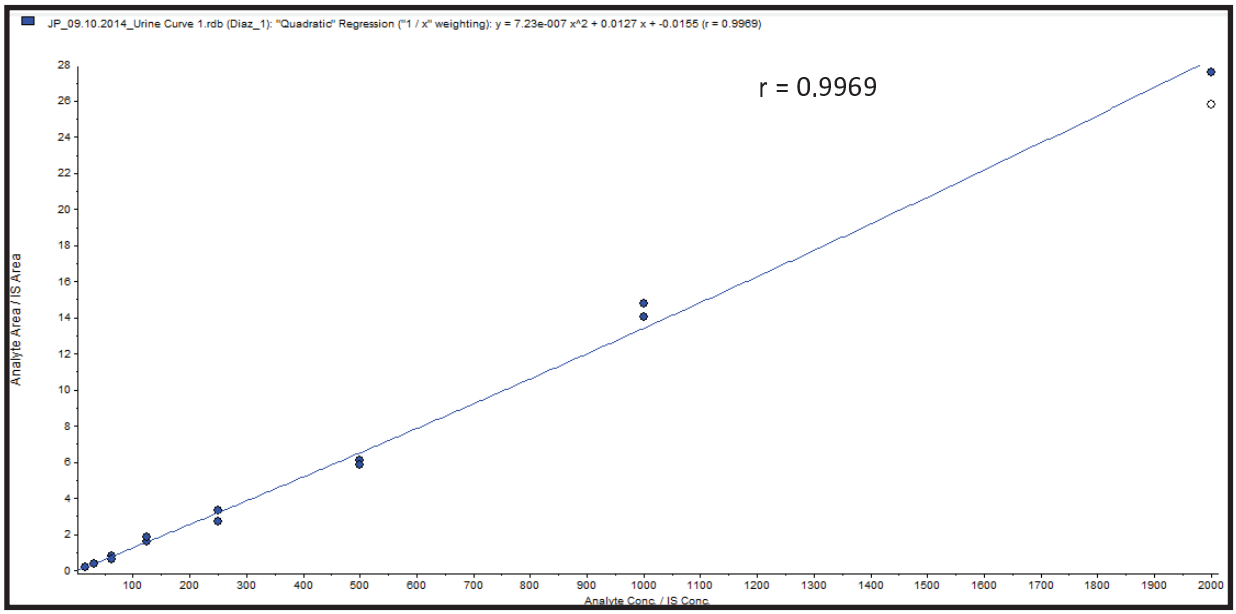


Figure C 16. Representative calibration curve for Diazepam: Validation 1, Day 1.

Table C 21.1. Diazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	17.1	1.81	10.5	109.9
31.3	S7	2 of 2	30.6	1.39	4.6	97.7
62.5	S6	2 of 2	57.3	8	14	91.7
125	S5	2 of 2	136	13.2	9.7	108.8
250	S4	2 of 2	234	33.1	14.1	93.7
500	S3	2 of 2	460	10.9	2.4	92
1000	S2	2 of 2	1070	37.9	3.5	107.1
2000	S1	1 of 2	1960	N/A	N/A	97.8

Table C 21.2. Summary of Diazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	13.6	1.18	8.7	87.4
45	L QC	2 of 2	47.1	0.99	2.1	104.7
800	M QC	2 of 2	769	7.7	1	96.1
1600	H QC	2 of 2	2090	514	24.6	130.6

Diazepam: Urine Validation 2, Day 2

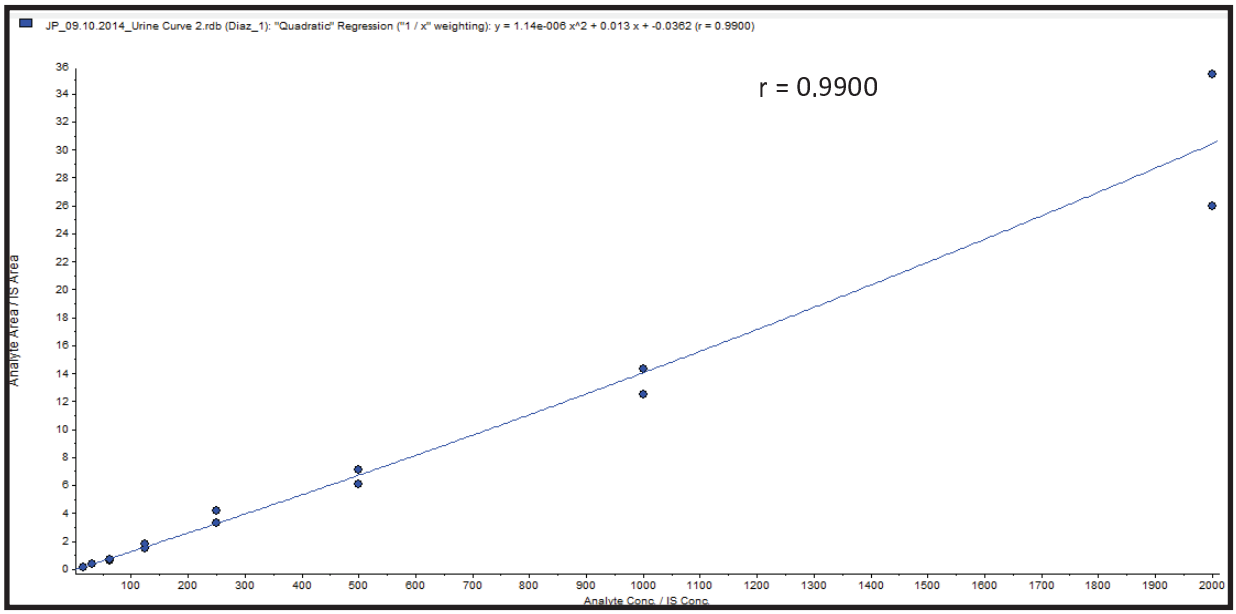


Figure C 17. Representative calibration curve for Diazepam: Validation 2, Day 2.

Table C 22.1. Diazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15	2.45	16.4	96
31.3	S7	2 of 2	32.2	0.6	1.9	102.9
62.5	S6	2 of 2	53.8	6.04	11.2	86
125	S5	2 of 2	131	17.2	13.1	105.2
250	S4	2 of 2	287	45	15.7	114.9
500	S3	2 of 2	490	49.5	10.1	98
1000	S2	2 of 2	958	82.8	8.6	95.8
2000	S1	2 of 2	2010	379	18.9	100.6

Table C 22.2. Summary of Diazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	12.5	0.73	5.8	80.1
45	L QC	2 of 2	43.8	0.82	1.9	97.4
800	M QC	2 of 2	858	193	22.5	107.2
1600	H QC	2 of 2	1750	381	21.7	109.6

<u>Diazepam</u>: Urine Validation 3, Day 3

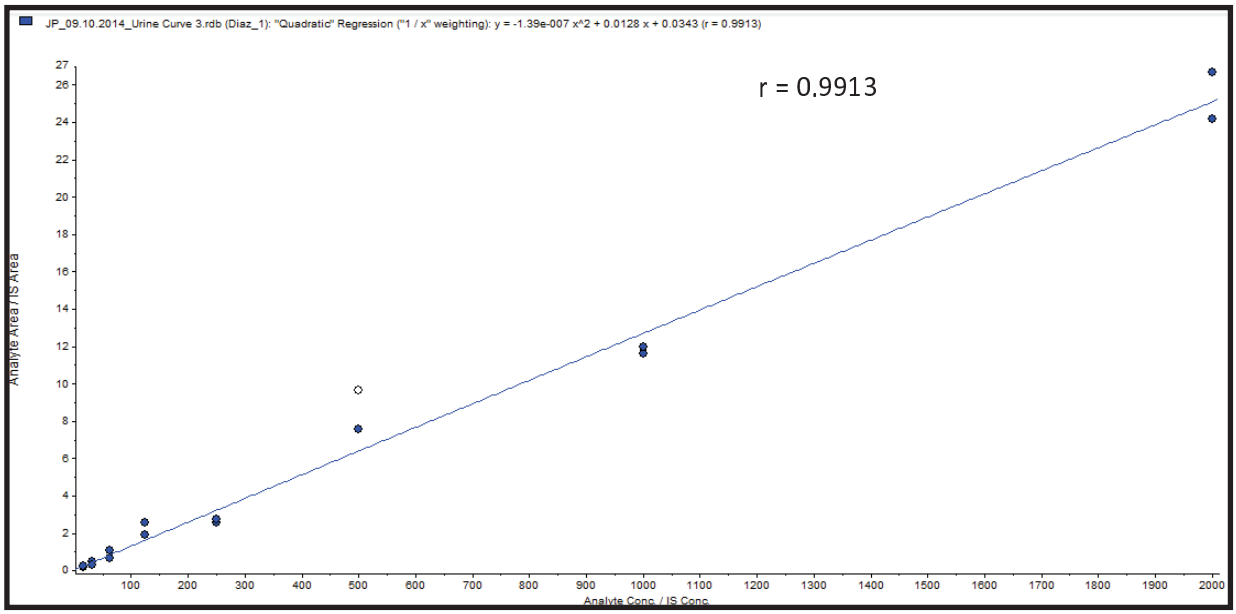


Figure C 18. Representative calibration curve for Diazepam: Validation 3, Day 3.

Table C 23.1. Diazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.2	2.53	19.2	84.4
31.3	S7	2 of 2	27.9	11.1	39.8	89
62.5	S6	2 of 2	64.4	22.2	34.5	103.1
125	S5	2 of 2	172	35.9	20.8	137.8
250	S4	2 of 2	206	11.3	5.5	82.4
500	S3	1 of 2	591	N/A	N/A	118.1
1000	S2	2 of 2	927	21.7	2.3	92.7
2000	S1	2 of 2	2030	144	7.1	101.4

Table C 23.2. Summary of Diazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	17.8	5.4	30.4	113.9
45	L QC	2 of 2	48	11.2	23.4	106.7
800	M QC	2 of 2	731	128	17.5	91.3
1600	H QC	2 of 2	2000	174	8.7	124.7

Table C 24.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	15.1	2.27	15.4	96.8
31.3	S7	6 of 6	30.2	4.36	15.4	96.5
62.5	S6	6 of 6	58.5	12.1	19.9	93.6
125	S5	5 of 6	147	22.1	14.5	117.3
250	S4	6 of 6	243	29.8	11.7	97
500	S3	5 of 6	514	30.2	6.2	102.7
1000	S2	6 of 6	985	47.5	4.8	98.5
2000	S1	5 of 6	2000	261	13	99.9

Table C 24.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	14.6	2.44	15	93.8
45	L QC	6 of 6	46.3	4.34	9.1	102.9
800	M QC	6 of 6	786	110	13.7	98.2
1600	H QC	6 of 6	1950	356	18.3	121.6

Flunitrazepam: Urine Validation 1, Day1

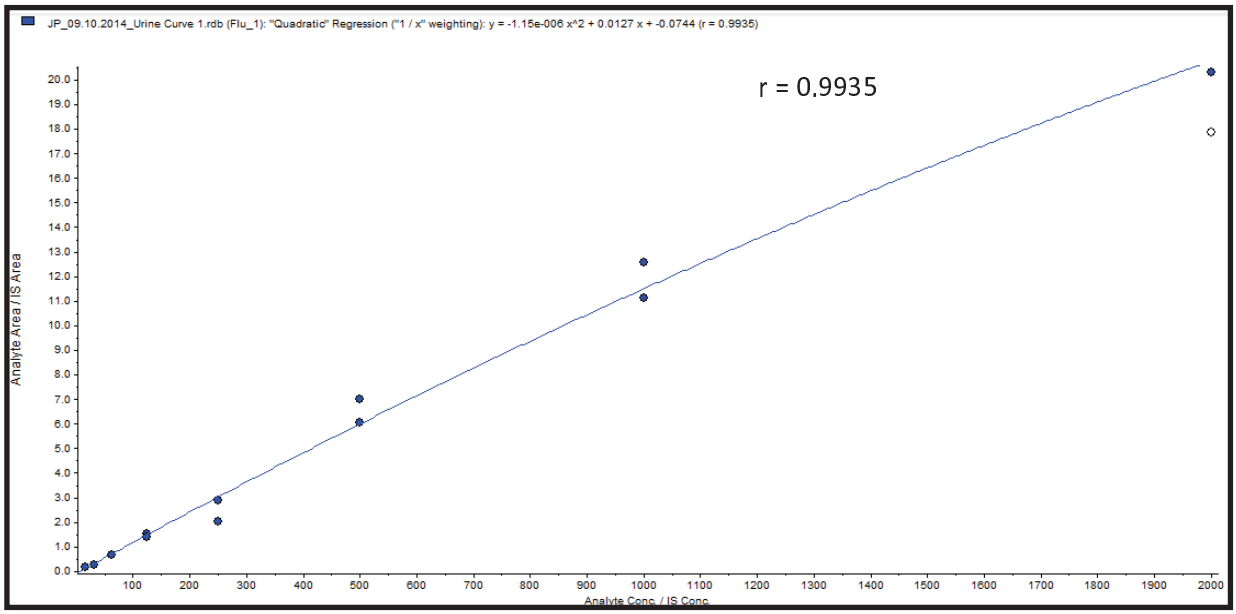


Figure C 19. Representative calibration curve for Flunitrazepam: Validation 1, Day 1.

Table C 25.1. Flunitrazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	20.3	1.29	6.3	130.4
31.3	S7	2 of 2	26.6	0.9	3.4	85
62.5	S6	2 of 2	58.8	0.02	0	94
125	S5	2 of 2	123	10.2	8.3	98.1
250	S4	2 of 2	204	51.4	25.3	81.4
500	S3	2 of 2	546	57.8	10.6	109.2
1000	S2	2 of 2	1030	101	9.7	103.4
2000	S1	1 of 2	1940	N/A	N/A	97.2

Table C 25.2. Summary of Flunitrazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	12.1	2.53	20.9	77.5
45	L QC	2 of 2	47.2	3.39	7.2	104.9
800	M QC	2 of 2	574	141	24.6	71.7
1600	H QC	2 of 2	1880	148	7.9	117.7

Flunitrazepam: Urine Validation 2, Day 2

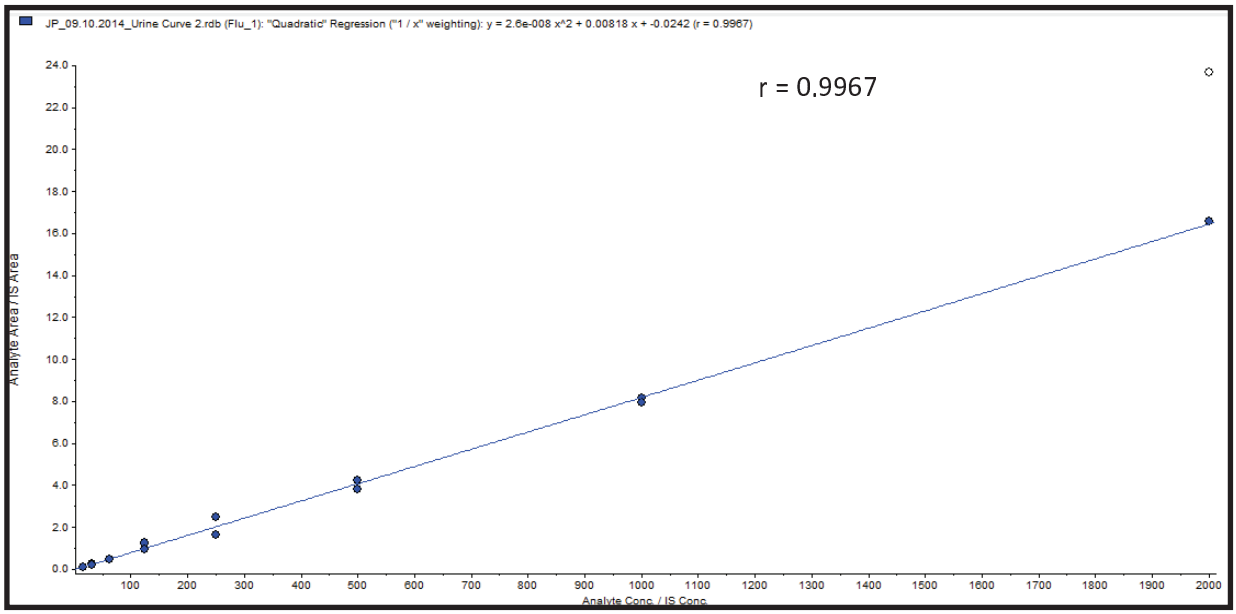


Figure C 20. Representative calibration curve for Flunitrazepam: Validation 2, Day 2.

Table C 26.1. Flunitrazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	14.6	0.04	0.3	93.4
31.3	S7	2 of 2	30.8	5.26	17.1	98.5
62.5	S6	2 of 2	61.7	1.91	3.1	98.7
125	S5	2 of 2	137	24.6	17.9	109.7
250	S4	2 of 2	255	73.1	28.7	101.9
500	S3	2 of 2	494	35.6	7.2	98.9
1000	S2	2 of 2	982	17.2	1.7	98.2
2000	S1	1 of 2	2020	N/A	N/A	100.9

Table C 26.2. Summary of Flunitrazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	16.9	5.09	30.1	108.4
45	L QC	2 of 2	37.4	10.8	28.8	83.2
800	M QC	2 of 2	942	334	35.4	117.7
1600	H QC	2 of 2	1980	388	19.6	123.8

<u>Flunitrazepam</u>: Urine Validation 3, Day 3

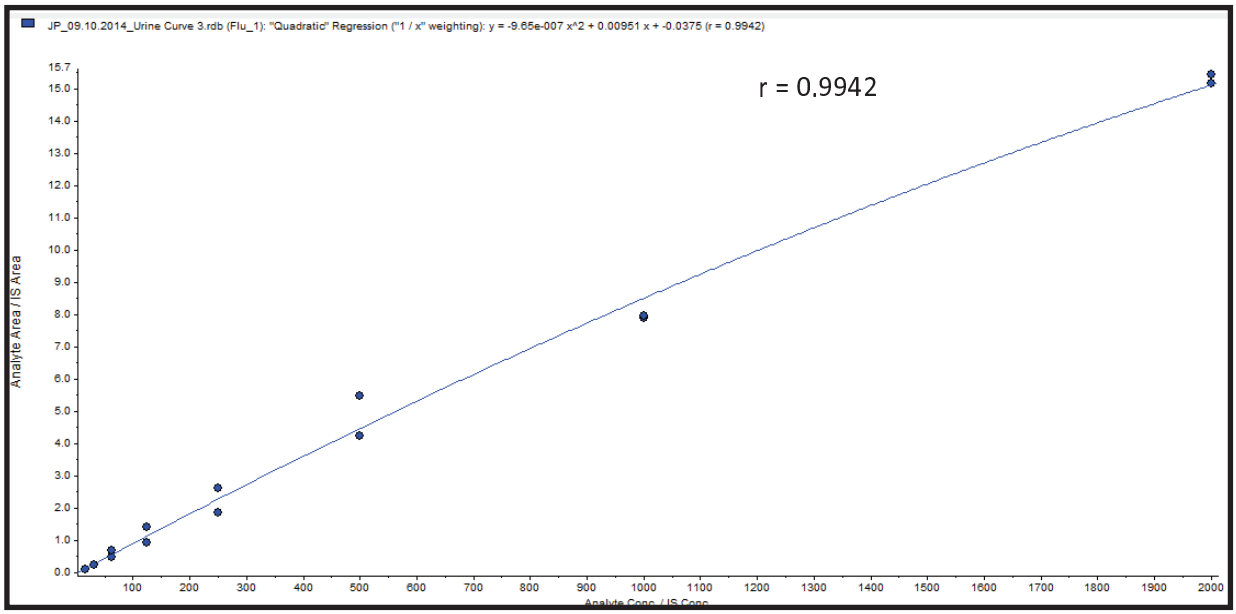


Figure C 21. Representative calibration curve for Flunitrazepam: Validation 3, Day 3.

Table C 27.1. Flunitrazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.4	1.4	9.1	98.5
31.3	S7	2 of 2	29.1	2	6.9	92.9
62.5	S6	2 of 2	64.8	16.3	25.1	103.8
125	S5	2 of 2	130	36.8	28.3	103.9
250	S4	2 of 2	245	60	24.5	97.9
500	S3	2 of 2	546	101	18.5	109.2
1000	S2	2 of 2	925	8.8	1	92.5
2000	S1	2 of 2	2030	34.5	1.7	101.7

Table C 27.2. Summary of Flunitrazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	17.2	2.77	16.1	109.9
45	L QC	2 of 2	40.8	7.4	18.1	90.6
800	M QC	2 of 2	708	204	28.8	88.5
1600	H QC	2 of 2	1830	10.2	0.6	114.2

Table C 28.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	16.8	0.91	5.2	107.4
31.3	S7	6 of 6	28.8	2.72	9.1	92.1
62.5	S6	6 of 6	61.8	6.06	9.4	98.8
125	S5	6 of 6	130	23.9	18.2	103.9
250	S4	6 of 6	234	61.5	26.2	93.7
500	S3	5 of 6	529	64.9	12.1	105.8
1000	S2	6 of 6	980	42.2	4.1	98
2000	S1	4 of 6	2000	34.5	1.7	99.9

Table C 28.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	15.4	3.46	22.4	98.6
45	L QC	6 of 6	41.8	7.19	18	92.9
800	M QC	6 of 6	741	226	29.6	92.7
1600	H QC	6 of 6	1900	182	9.3	118.6

<u>Lorazepam</u>: Urine Validation 1, Day1

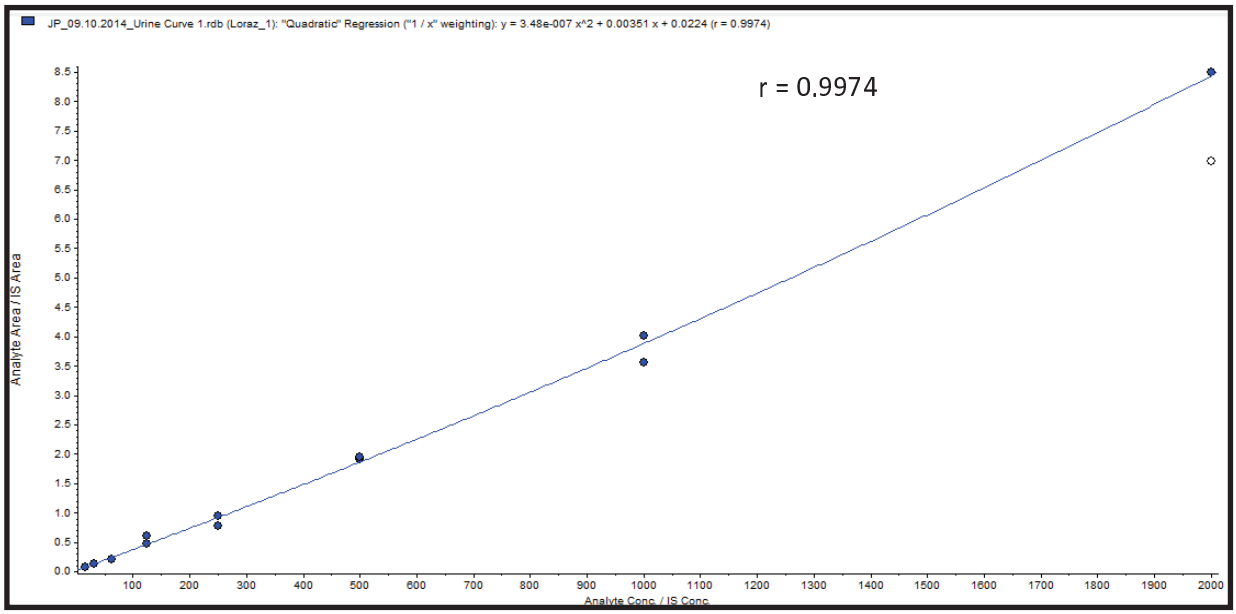


Figure C 22. Representative calibration curve for Lorazepam: Validation 1, Day 1.

Table C 29.1. Lorazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.6	0.79	5.1	99.9
31.3	S7	2 of 2	31.7	3.39	10.7	101.1
62.5	S6	2 of 2	54.2	0.73	1.3	86.7
125	S5	2 of 2	146	24.9	17.1	116.6
250	S4	2 of 2	234	33.3	14.3	93.4
500	S3	2 of 2	519	5.99	1.2	103.8
1000	S2	2 of 2	978	75.9	7.8	97.8
2000	S1	1 of 2	2010	N/A	N/A	100.6

Table C 29.2. Summary of Lorazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	6.2	3.78	61	39.7
45	L QC	2 of 2	42.1	13.7	32.6	93.5
800	M QC	2 of 2	718	3.95	0.6	89.8
1600	H QC	2 of 2	1950	317	16.2	122

Lorazepam: Urine Validation 2, Day 2

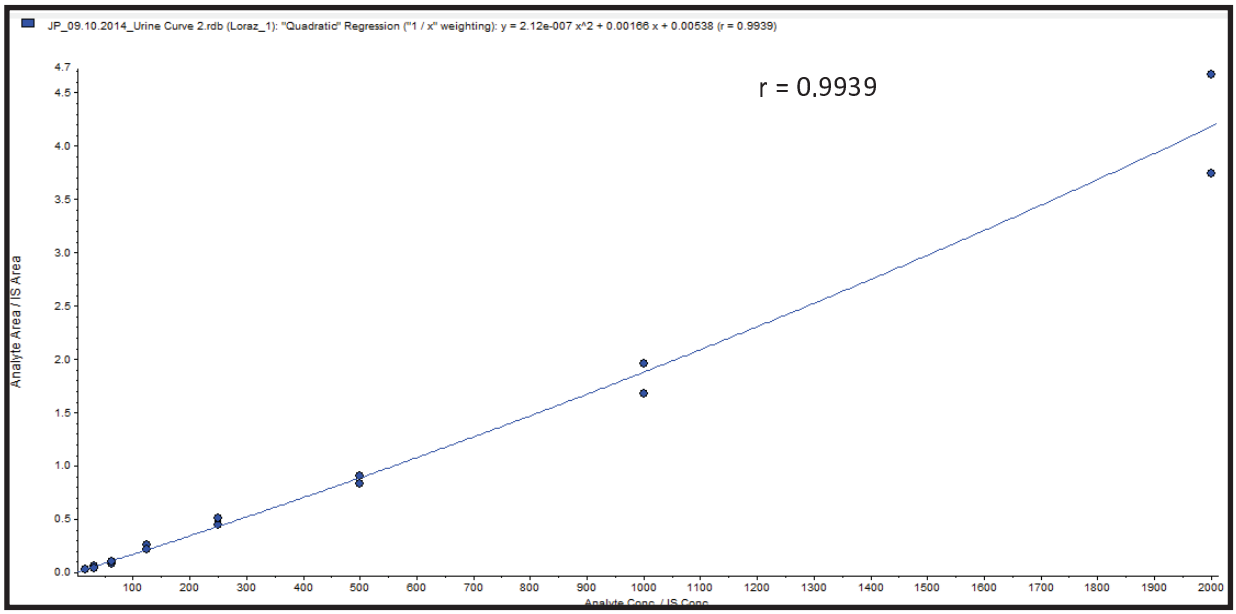


Figure C 23. Representative calibration curve for Lorazepam: Validation 2, Day 2.

Table C 30.1. Lorazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.3	1.6	9.8	104.4
31.3	S7	2 of 2	29	9.47	32.7	92.5
62.5	S6	2 of 2	53.6	8.76	16.3	85.7
125	S5	2 of 2	139	20.1	14.4	111.4
250	S4	2 of 2	275	27.2	9.9	110.1
500	S3	2 of 2	489	27.4	5.6	97.7
1000	S2	2 of 2	972	94.5	9.7	97.2
2000	S1	2 of 2	2010	261	13	100.4

Table C 30.2. Summary of Lorazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	9.65	7.76	80.4	61.9
45	L QC	2 of 2	43.6	8.17	18.8	96.8
800	M QC	2 of 2	874	150	17.2	109.2
1600	H QC	2 of 2	1710	210	12.3	106.6

Lorazepam: Urine Validation 3, Day 3

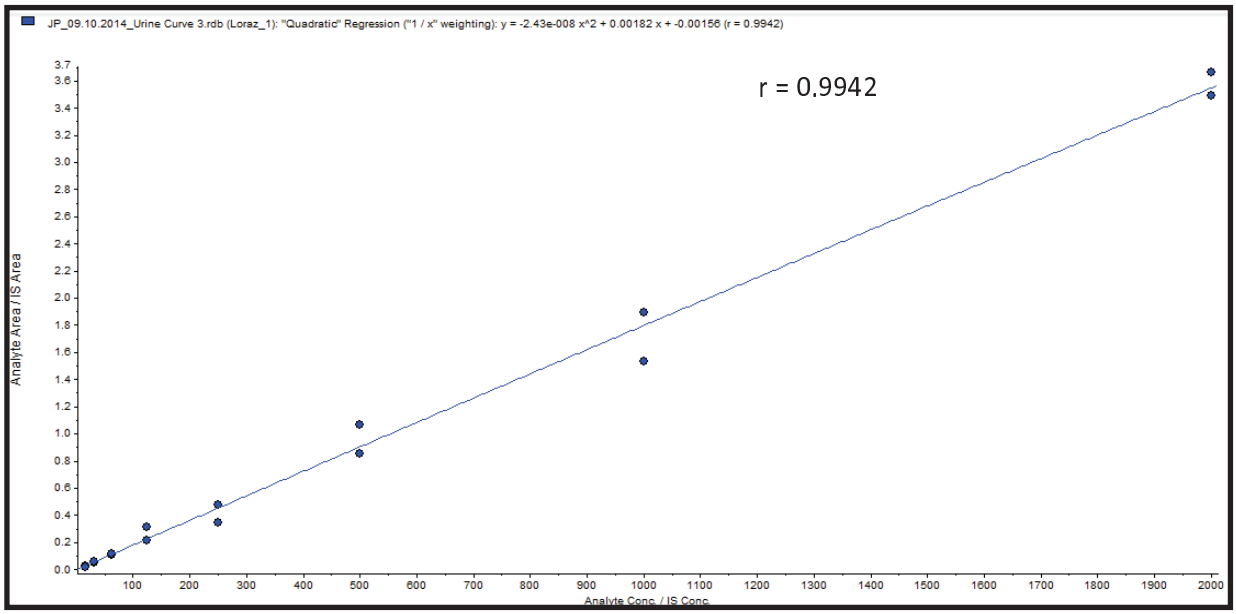


Figure C 24. Representative calibration curve for Lorazepam: Validation 3, Day 3.

Table C 31.1. Lorazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.4	4.61	34.3	86
31.3	S7	2 of 2	32.3	2.65	8.2	103.2
62.5	S6	2 of 2	62	2.2	3.6	99.2
125	S5	2 of 2	147	37	25.2	117.6
250	S4	2 of 2	229	50.7	22.1	91.6
500	S3	2 of 2	531	84.8	16	106.1
1000	S2	2 of 2	952	145	15.2	95.2
2000	S1	2 of 2	2020	69.8	3.5	100.9

Table C 31.2. Summary of Lorazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	14.8	5.05	34.1	95.1
45	L QC	2 of 2	49.6	12.3	24.7	110.3
800	M QC	2 of 2	786	25	3.2	98.2
1600	H QC	2 of 2	1770	291	16.5	110.3

Table C 32.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	15.1	2.33	16.4	96.8
31.3	S7	6 of 6	31	5.17	17.2	99
62.5	S6	6 of 6	56.6	3.9	7.1	90.5
125	S5	6 of 6	144	27.3	18.9	115.2
250	S4	6 of 6	246	37.1	15.4	98.4
500	S3	5 of 6	513	39.4	7.6	102.6
1000	S2	6 of 6	967	105	10.9	96.7
2000	S1	5 of 6	2010	165	8.2	100.6

Table C 32.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	10.2	5.53	58.5	65.6
45	L QC	6 of 6	45.1	11.4	25.4	100.2
800	M QC	6 of 6	792	59.6	7	99.1
1600	H QC	6 of 6	1810	273	15	113

Nitrazepam: Urine Validation 1, Day1

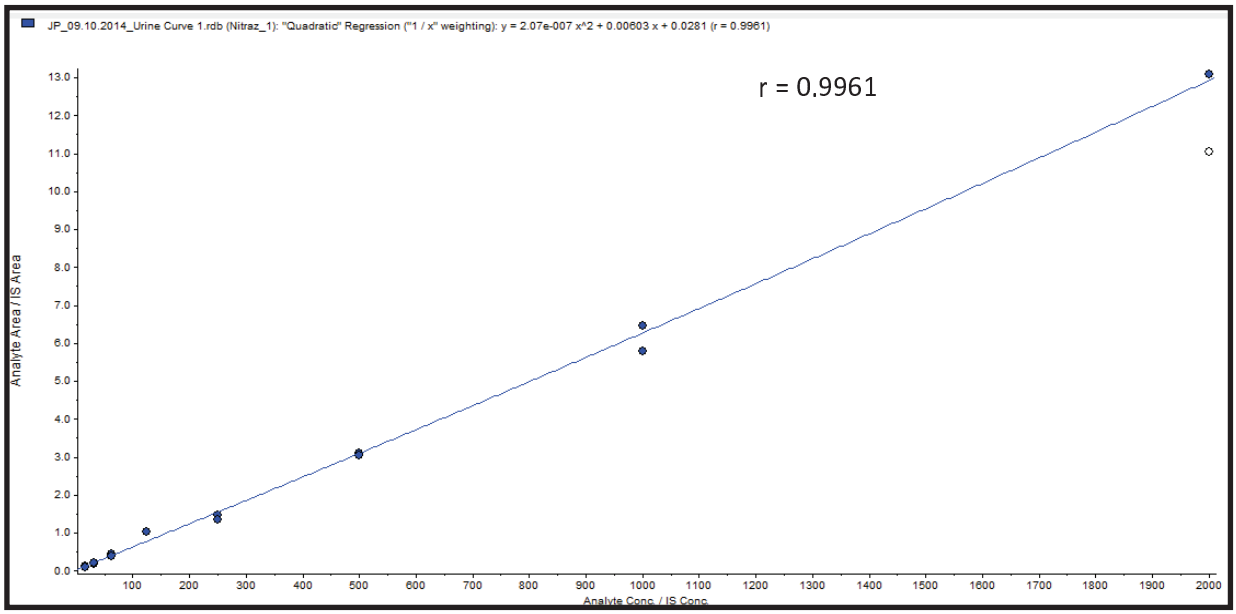


Figure C 25. Representative calibration curve for Nitrazepam: Validation 1, Day 1.

Table C 33.1. Nitrazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13	3.23	24.9	83.2
31.3	S7	2 of 2	29.1	1.98	6.8	93
62.5	S6	2 of 2	63.8	6.93	10.9	102.1
125	S5	2 of 2	166	1.47	0.9	133
250	S4	2 of 2	227	13.6	6	91
500	S3	2 of 2	496	7.06	1.4	99.1
1000	S2	2 of 2	977	72.6	7.4	97.7
2000	S1	1 of 2	2020	N/A	N/A	101.2

Table C 33.2. Summary of Nitrazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	11	5.61	51.1	70.3
45	L QC	2 of 2	43.2	6.69	15.5	96
800	M QC	2 of 2	721	5.58	0.8	90.1
1600	H QC	2 of 2	2070	569	27.5	129.2

Nitrazepam: Urine Validation 2, Day 2

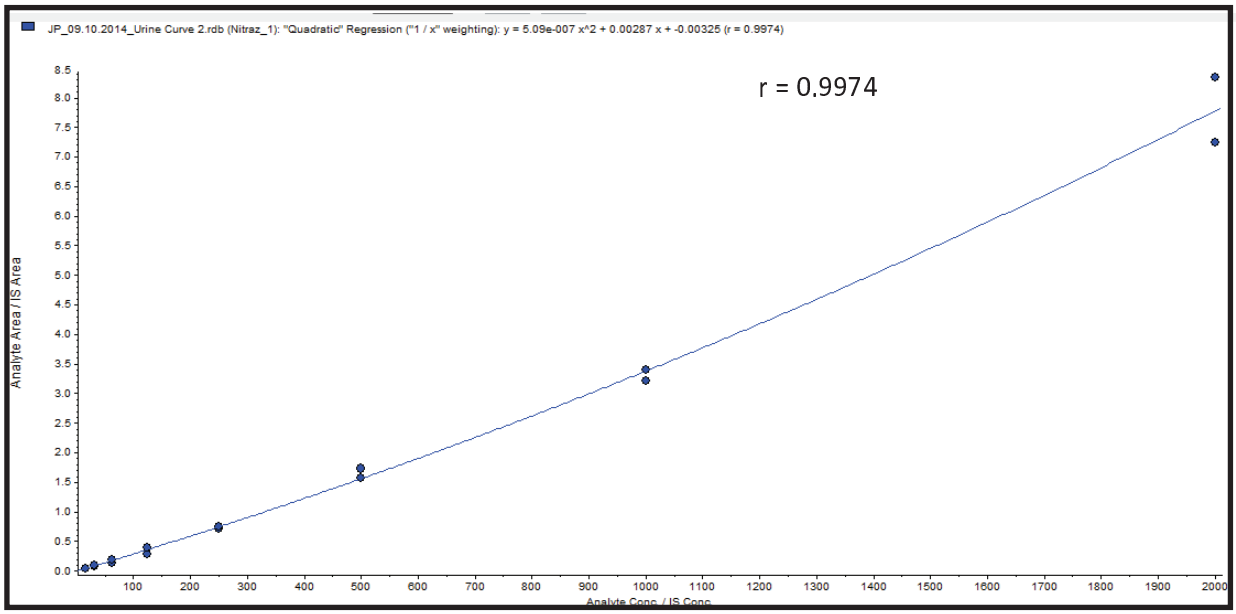


Figure C 26. Representative calibration curve for Nitrazepam: Validation 2, Day 2.

Table C 34.1. Nitrazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	16.4	1.77	10.8	105
31.3	S7	2 of 2	33.3	1.74	5.2	106.3
62.5	S6	2 of 2	57.6	13.5	23.5	92.1
125	S5	2 of 2	117	22.3	19	94
250	S4	2 of 2	246	8.57	3.5	98.3
500	S3	2 of 2	528	31.5	6	105.5
1000	S2	2 of 2	983	34.5	3.5	98.3
2000	S1	2 of 2	2000	160	8	100.1

Table C 34.2. Summary of Nitrazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	14.5	3.57	24.7	92.7
45	L QC	2 of 2	42	0.81	1.9	93.4
800	M QC	2 of 2	768	303	39.4	96.1
1600	H QC	2 of 2	1730	200	11.6	108.1

Nitrazepam: Urine Validation 3, Day 3

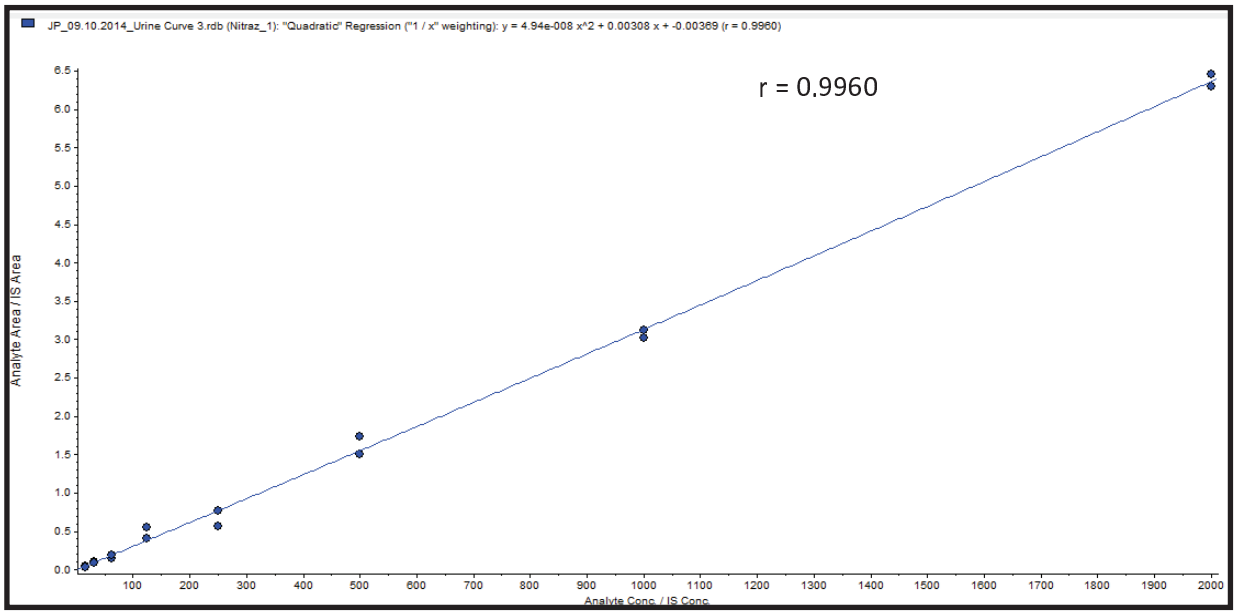


Figure C 27. Representative calibration curve for Nitrazepam: Validation 3, Day 3.

Table C 35.1. Nitrazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	14.7	5.34	36.3	94.2
31.3	S7	2 of 2	32.2	5.42	16.8	103
62.5	S6	2 of 2	55.3	11.1	20.2	88.4
125	S5	2 of 2	156	34.6	22.2	124.8
250	S4	2 of 2	216	45.6	21.1	86.4
500	S3	2 of 2	522	52.2	10	104.4
1000	S2	2 of 2	982	22.3	2.3	98.2
2000	S1	2 of 2	2010	32.9	1.6	100.3

Table C 35.2. Summary of Nitrazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	21.4	4.37	20.4	137
45	L QC	2 of 2	43.9	13.7	31.2	97.5
800	M QC	2 of 2	800	86.6	10.8	100
1600	H QC	2 of 2	1630	337	20.7	101.8

Table C 36.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	6 of 6	14.7	3.45	24	94.1
31.3	S7	6 of 6	31.5	3.05	9.6	100.8
62.5	S6	6 of 6	58.9	10.5	18.2	94.2
125	S5	6 of 6	147	19.5	14	117.3
250	S4	6 of 6	230	22.6	10.2	91.9
500	S3	6 of 6	515	30.3	5.8	103
1000	S2	6 of 6	981	43.1	4.4	98.1
2000	S1	5 of 6	2010	96.4	4.8	100.5

Table C 36.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	6 of 6	15.6	4.52	32.1	100
45	L QC	6 of 6	43	7.07	16.2	95.7
800	M QC	6 of 6	763	132	17	95.4
1600	H QC	6 of 6	1810	369	19.9	113

<u>Oxazepam</u>: Urine Validation 1, Day1

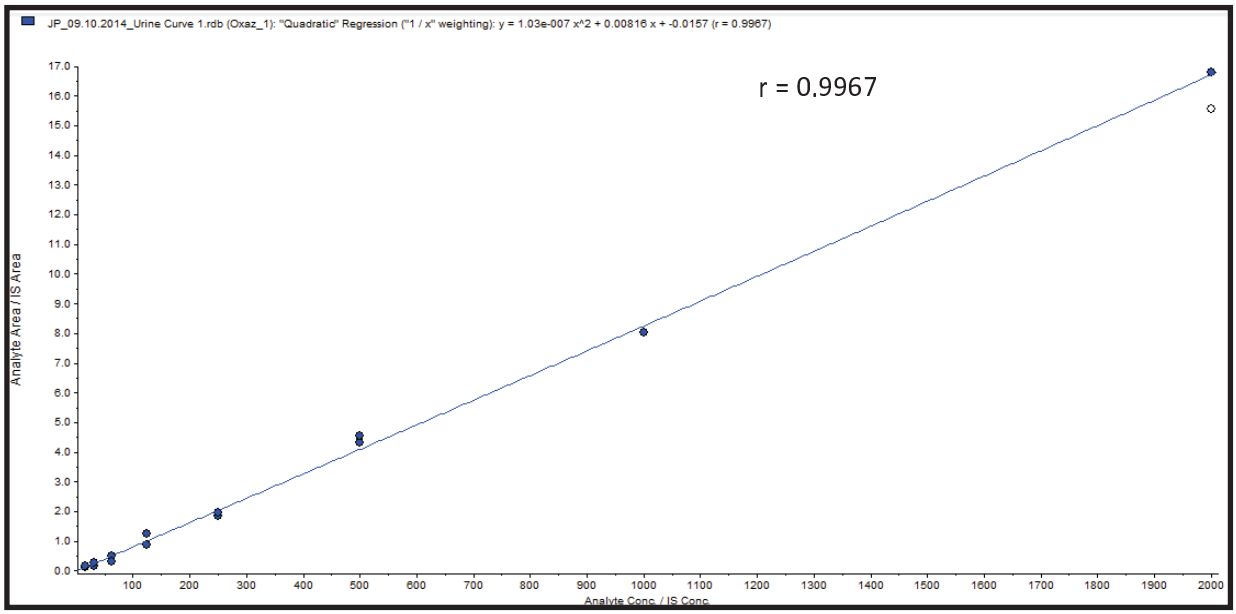


Figure C 28. Representative calibration curve for Oxazepam: Validation 1, Day 1.

Table C 37.1. Oxazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	18.6	3.11	16.7	119
31.3	S7	2 of 2	29.8	8.56	28.7	95.2
62.5	S6	2 of 2	50.4	16.3	32.2	80.7
125	S5	2 of 2	131	34	25.8	105.2
250	S4	2 of 2	235	9.66	4.1	93.9
500	S3	2 of 2	541	17.8	3.3	108.3
1000	S2	2 of 2	973	0.96	0.1	97.3
2000	S1	1 of 2	2010	N/A	N/A	100.5

Table C 37.2. Summary of Oxazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	12.6	2.19	17.4	80.7
45	L QC	2 of 2	46.4	15.7	33.8	103.2
800	M QC	2 of 2	764	12.5	1.6	95.5
1600	H QC	2 of 2	2100	435	20.7	131.3

<u>Oxazepam</u>: Urine Validation 2, Day 2

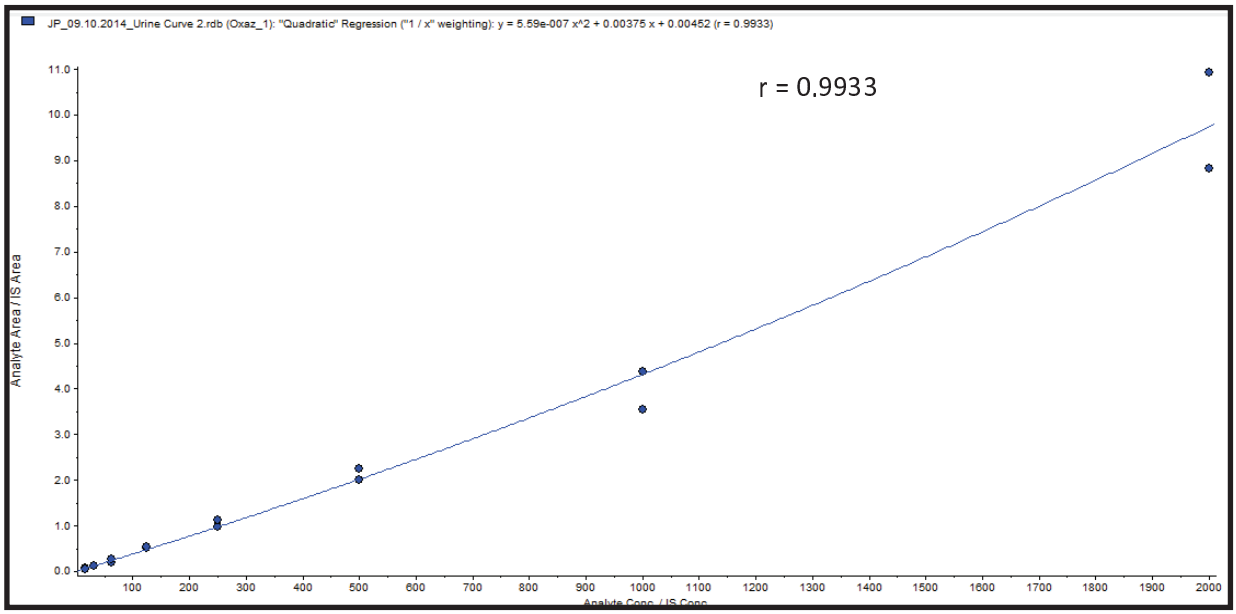


Figure C 29. Representative calibration curve for Oxazepam: Validation 2, Day 2.

Table C 38.1. Oxazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	13.7	3.09	22.5	88.1
31.3	S7	2 of 2	31.2	1.32	4.2	99.6
62.5	S6	2 of 2	59.6	14.9	24.9	95.4
125	S5	2 of 2	138	6.9	5	110.1
250	S4	2 of 2	269	25	9.3	107.7
500	S3	2 of 2	525	36.1	6.9	105
1000	S2	2 of 2	926	120	13	92.6
2000	S1	2 of 2	2020	246	12.2	101

Table C 38.2. Summary of Oxazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	13.3	3.47	26.1	85.3
45	L QC	2 of 2	46.4	5.17	11.1	103.1
800	M QC	2 of 2	850	84.3	9.9	106.3
1600	H QC	2 of 2	1780	329	18.5	110.9

Oxazepam: Urine Validation 3, Day 3

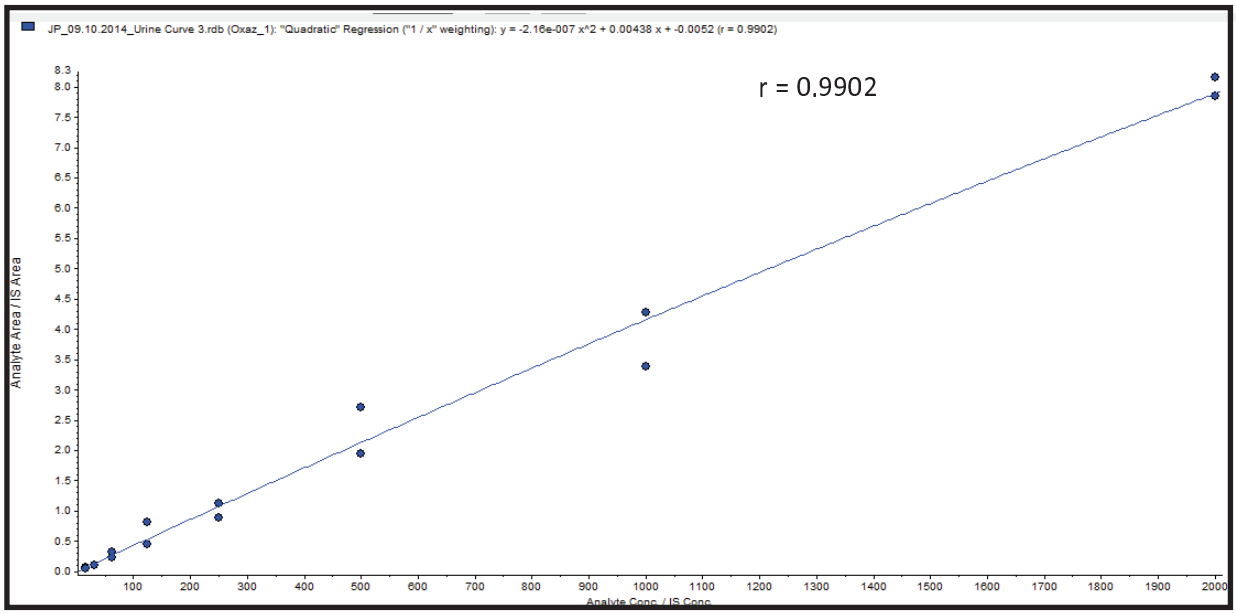


Figure C 30. Representative calibration curve for Oxazepam: Validation 3, Day 3.

Table C 39.1. Oxazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.2	3.99	26.3	97.4
31.3	S7	2 of 2	26.9	0.26	1	86.1
62.5	S6	2 of 2	64.1	13.5	21	102.6
125	S5	2 of 2	146	59.4	40.6	116.9
250	S4	2 of 2	235	39.6	16.8	94.2
500	S3	2 of 2	548	130	23.7	109.6
1000	S2	2 of 2	917	158	17.3	91.7
2000	S1	2 of 2	2030	61.5	3	101.7

Table C 39.2. Summary of Oxazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	15.7	3.31	21.1	100.8
45	L QC	2 of 2	57	15.1	26.5	126.6
800	M QC	2 of 2	810	2.32	0.3	101.2
1600	H QC	2 of 2	1960	380	19.4	122.6

Table C 40.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.8	3.4	21.8	101.5
31.3	S7	2 of 2	29.3	3.38	11.3	93.6
62.5	S6	2 of 2	58	14.9	26.1	92.9
125	S5	2 of 2	138	33.4	23.8	110.7
250	S4	2 of 2	246	24.7	10.1	98.6
500	S3	2 of 2	538	61.4	11.3	107.6
1000	S2	2 of 2	939	93.1	10.1	93.9
2000	S1	2 of 2	2020	154	7.6	101.1

Table C 40.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	13.9	2.99	21.5	88.9
45	L QC	2 of 2	49.9	12	23.8	111
800	M QC	2 of 2	808	33	3.9	101
1600	H QC	2 of 2	1950	381	19.5	121.6

Temazepam: Urine Validation 1, Day1

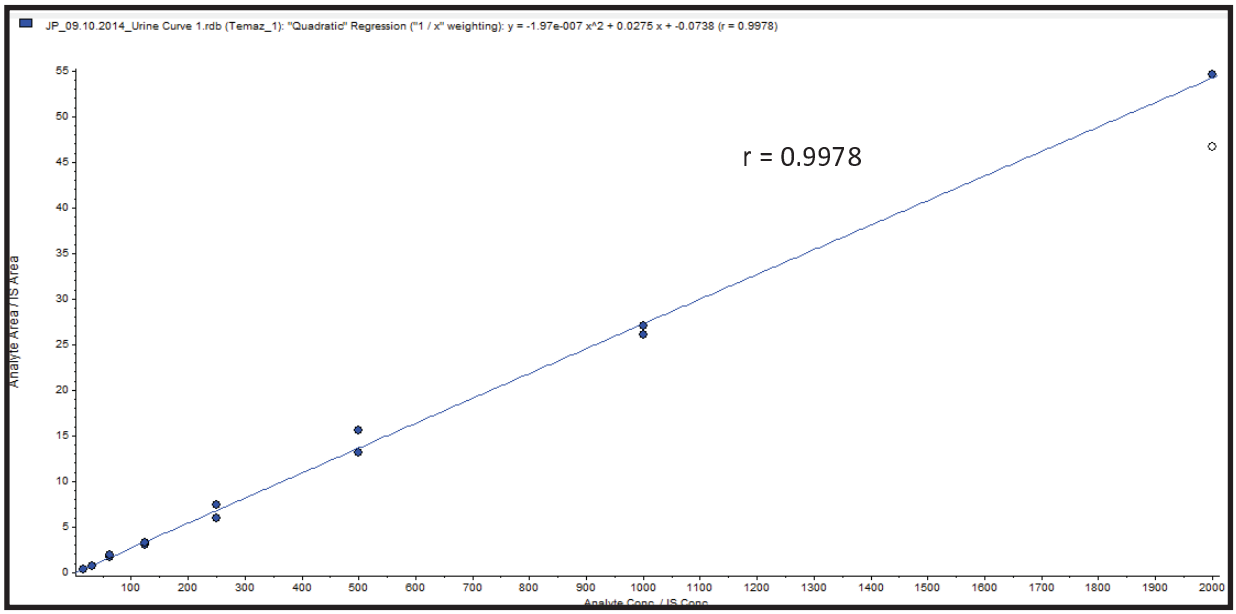


Figure C 31. Representative calibration curve for Temazepam: Validation 1, Day 1.

Table C 41.1. Temazepam Calibration Standards Accuracy and Precision – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	15.2	0.23	1.5	97.6
31.3	S7	2 of 2	30.1	0.81	2.7	96
62.5	S6	2 of 2	69.9	7.16	10.2	111.8
125	S5	2 of 2	117	6.72	5.8	93.5
250	S4	2 of 2	245	36.8	15	98
500	S3	2 of 2	526	64.1	12.2	105.1
1000	S2	2 of 2	974	25.8	2.6	97.4
2000	S1	1 of 2	2010	N/A	N/A	100.7

Table C 41.2. Summary of Temazepam intra-validation quality control standards – Validation 1

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	12.8	1.53	11.9	82.2
45	L QC	2 of 2	36.9	2.19	5.9	82.1
800	M QC	2 of 2	609	90.9	14.9	76.1
1600	H QC	2 of 2	1970	353	17.9	122.9

Temazepam: Urine Validation 2, Day 2

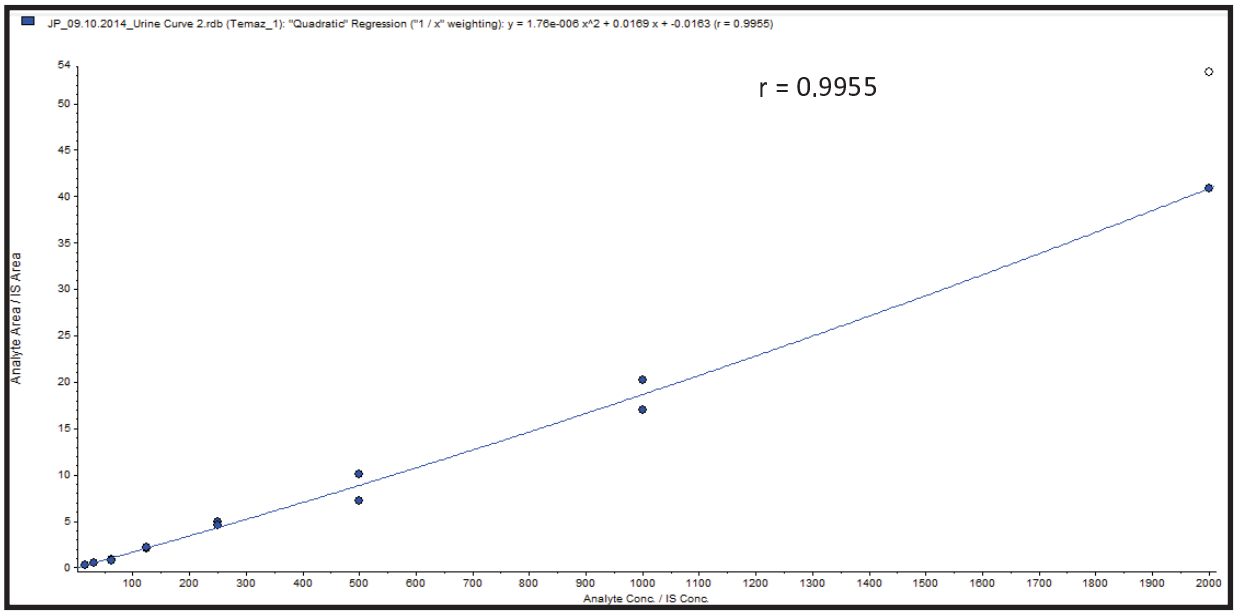


Figure C 32. Representative calibration curve for Temazepam: Validation 2, Day 2.

Table C 42.1. Temazepam Calibration Standards Accuracy and Precision – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	18	1.2	6.7	115.4
31.3	S7	2 of 2	30.9	3.1	10	98.7
62.5	S6	2 of 2	49.6	2.11	4.3	79.3
125	S5	2 of 2	123	4.43	3.6	98.8
250	S4	2 of 2	276	13.1	4.8	110.3
500	S3	2 of 2	487	105	21.5	97.4
1000	S2	2 of 2	998	112	11.2	99.8
2000	S1	1 of 2	2000	N/A	N/A	100.1

Table C 42.2. Summary of Temazepam intra-validation quality control standards – Validation 2

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	13.8	1.06	7.7	88.3
45	L QC	2 of 2	42.6	5.36	12.6	94.6
800	M QC	2 of 2	871	32	3.7	108.9
1600	H QC	2 of 2	1890	432	22.9	118.1

<u>Temazepam</u>: Urine Validation 3, Day 3

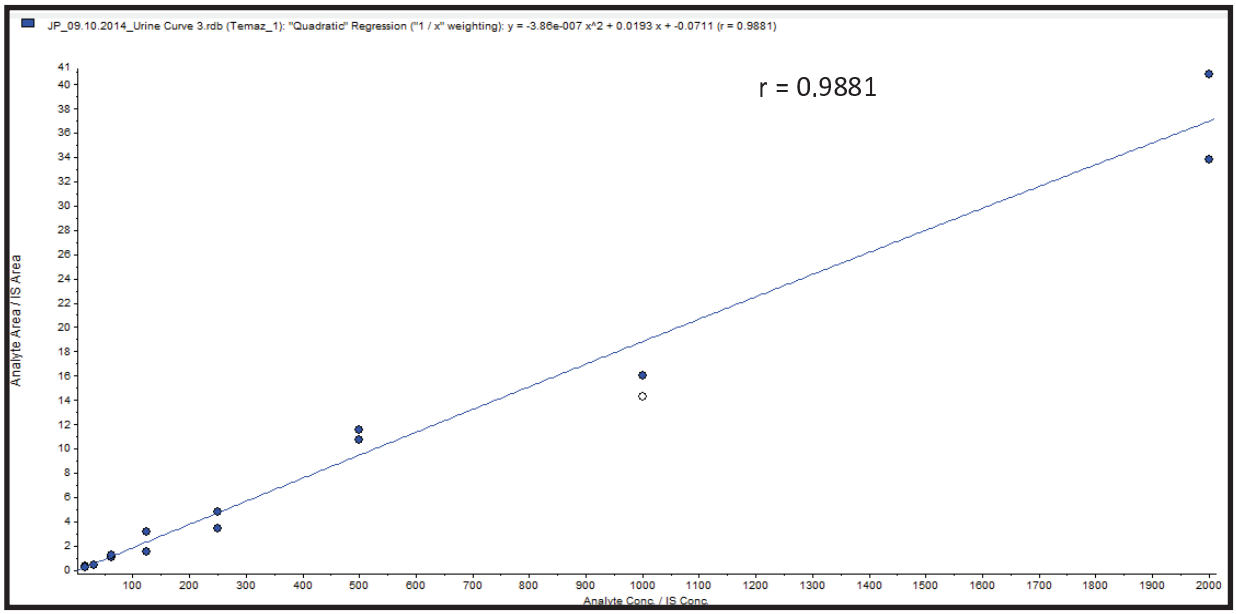


Figure C 33. Representative calibration curve for Temazepam: Validation 3, Day 3.

Table C 43.1. Temazepam Calibration Standards Accuracy and Precision – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	18	3.18	17.6	115.5
31.3	S7	2 of 2	25.8	0.04	0.2	82.4
62.5	S6	2 of 2	64.9	6.72	10.3	103.9
125	S5	2 of 2	125	62.1	49.8	99.7
250	S4	2 of 2	219	52.8	24.2	87.4
500	S3	2 of 2	587	30.4	5.2	117.5
1000	S2	1 of 2	851	N/A	N/A	85.1
2000	S1	2 of 2	2020	281	13.9	101

Table C 43.2. Summary of Temazepam intra-validation quality control standards – Validation 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	19.8	5.36	27.1	126.9
45	L QC	2 of 2	38.1	5.29	13.9	84.7
800	M QC	2 of 2	738	61.1	8.3	92.3
1600	H QC	2 of 2	1890	253	13.4	118.2

Table C 44.1. Overall Summary of Calibration Standard Accuracy and Precision: Validation 1 – 3

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	S8	2 of 2	17.1	1.54	8.6	109.5
31.3	S7	2 of 2	28.9	1.32	4.3	92.4
62.5	S6	2 of 2	61.5	5.33	8.3	98.3
125	S5	2 of 2	122	24.4	19.7	97.3
250	S4	2 of 2	246	34.2	14.6	98.6
500	S3	2 of 2	533	66.4	13	106.6
1000	S2	1 of 2	941	68.8	6.9	94.1
2000	S1	2 of 2	2010	281	13.9	100.6

Table C 44.2. Overall Quality Control Accuracy and Precision Estimation

Expected Concentration (ng/mL)	Sample Name	n	Mean	Standard Deviation	%CV	%Accuracy
15.6	LLOQ	2 of 2	15.5	2.65	15.6	99.1
45	L QC	2 of 2	39.2	4.28	10.8	87.1
800	M QC	2 of 2	740	61.3	9	92.4
1600	H QC	2 of 2	1920	346	18.1	119.7

Here follows a list of %Accuracy values that did not fall within the acceptance criteria values for calibration standards and quality controls.

Table C 45 %Accuracy values that did not fit acceptance criteria and an indication of the amount of percent with which it was too high or too low to get to either the lowest or highest accepted percentage.

Analyte	Validation Batch	STD/QC Fail	%Accuracy	+/- % out*
Nortriptyline	Validation 1	S7	83.15297	+ 2
		LLOQ	77.35549	+ 3
		H QC	123.4583	-8
	Validation 2	L QC	122.8907	- 8
		M QC	117.1765	- 2
	Validation 3	L QC	116.1181	- 1
	Overall	L QC	117.5608	- 3
		H QC	116.4778	- 1
Amitriptyline	Validation 1	LLOQ	70.71903	+ 9
		H QC	138.9048	-24
	Validation 2	L QC	117.3814	- 2
		H QC	124.1232	- 9
	Validation 3	S7	81.65878	+ 3
		S5	122.6768	-8
		LLOQ	126.613	- 12
		L QC	120.6945	- 6
	Overall	L QC	119.0319	- 4
		H QC	123.4872	- 8
Citalopram	Validation 1	H QC	122.5513	- 8
	Validation 2	S6	82.60654	+ 2
	Validation 3	S6	80.08125	+ 3
		S5	117.5554	-3
		H QC	121.8115	- 7
	Overall	H QC	119.2991	- 4
Alprazolam	Validation 1	S8	69.88678	+ 15
		S5	123.7549	- 9
		LLOQ	55.14636	+ 25
		L QC	80.32515	+ 5
	Validation 2	S8	79.52356	+ 5
	Validation 3	L QC	138.3444	- 23
		H QC	116.5906	- 2
Clonazepam	Validation 1	S6	79.31911	+ 6
		LLOQ	77.40062	+ 8
		H QC	129.1636	-14
	Validation 2	M QC	120.6151	- 6
	Validation 3	LLOQ	68.60229	+ 13
		L QC	118.6464	-4
		H QC	121.3511	- 6
	Overall	H QC	120.8071	- 6
Diazepam	Validation 1	H QC	130.5968	- 16
	Validation 3	H QC	124.7462	- 10

	Overall	H QC	121.637	- 7
Flunitrazepam	Validation 1	S4	81.40896	+ 3
		LLOQ	77.47763	+ 3
		M QC	71.7212	+ 12
		H QC	117.6976	- 3
	Validation 2	L QC	83.2024	+ 2
		M QC	117.7373	- 3
		H QC	123.7858	- 9
	Overall	H QC	118.5673	- 4
Lorazepam	Validation 1	S5	116.582	- 2
		LLOQ	39.73403	+ 40
		H QC	122.0138	-7
	Validation 2	LLOQ	61.86232	+ 18
Nitrazepam	Validation 1	LLOQ	70.28824	+ 10
		H QC	129.1709	- 14
	Validation 3	LLOQ	137.0451	-17
Oxazepam	Validation 1	S6	80.69868	+ 4
		LLOQ	80.67769	+ 4
		H QC	131.3226	-16
	Validation 3	L QC	126.6052	- 12
		H QC	122.5904	- 8
	Overall	H QC	121.6168	- 8
Temazepam	Validation 1	L QC	82.11079	+ 3
		M QC	76.10478	+ 9
		H QC	122.8503	-12
	Validation 2	S6	79.29017	+ 6
		H QC	118.0526	-3
	Validation 3	S7	82.38097	+ 3
		L QC	126.8632	- 12
		H QC	118.2283	- 3
	Overall	H QC	119.7104	- 5

*Rounding up to get to lowest or highest accepted %Accuracy values

D: Human Research Ethics Committee Approval

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6492 • **Facsimile** [021] 406 6411
Email: Sumayah.ariefdien@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

22 July 2014

HREC/REF: 547/2014

A/Prof P Smith
Pharmacology
K-45
K-floor
OMB

Dear A/Prof Smith

Project Title: DEVELOPMENT AND VALIDATION OF A METHOD FOR THE QUANTIFICATION OF BENZODIAZEPINES, OPIATES AND ANTIDEPRESSANTS IN WHOLE BLOOD, SERUM AND URINE BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (MPhil-candidate-J Pieters)

Thank you submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It is a pleasure to inform you that the HREC has **formally approved** the above mentioned study.

Approval is granted for one year until the 30 July 2015.

Please submit a progress form, using the standardised Annual Report Form, if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.

We acknowledge that the following student:- Janke Pieters is also involved in this project.

Please note that the on-going ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the HREC REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.

Hrec/ref:547/2014

E: Repository Registration of Specimens from Medico-Lega Autopsies Form

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
Email: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/research/humanethics/forms

08 May 2014

REF NO: R016/2014

Prof L Martin
Division of Forensic Medicine & Toxicology
Falmouth Building
Entrance 3
Level 1

Dear Prof Martin

PROJECT TITLE: ***Repository of Pathology Specimens from Medico-Legal Autopsies***

Thank you for your submission to the Faculty of Health Sciences Human Research Ethics Committee.

The HREC has **approved** the registration of your repository.

Please Note: All research, including that undertaken for a master's or doctoral degree, using registered databases, registries and repositories, requires submission as a new study. It requires an application form (FHS013) and a protocol which has undergone departmental review. The study will receive its own HREC REF number which will be linked to the main database or repository.

The registration of this database is valid until **30 May 2017.**

Please quote the HREC REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

F: Certificates of Analysis for Reference and Internal Standard

Cerilliant
Analytical Reference Standards
a SIGMA-ALDRICH business

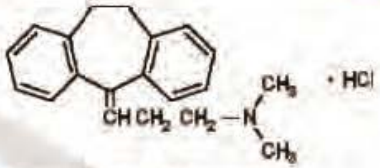
A-923
FN102912-01
Revision 0
Page 1 of 6
Product of USA

ISO GUIDE 34
ISO/IEC 17025
ISO 13485
ISO 9001
GMP/GLP

Certificate of Analysis

Amitriptyline Hydrochloride

Catalog Number:	A-923
Solution Lot:	FN102912-01
Expiration Date:	December 2017
Solvent:	Methanol (LC-MS Chromasolv®)
Volume per Ampule:	Not less than 1 mL
Storage:	Store unopened in freezer.
Shipping:	Ambient. See Stability Section.
Intended Use:	For R&D analytical purposes only. Not suitable for human or animal consumption.
Safety:	Flammable, Poison



- Expiration Date has been established through real time stability studies.
- Ampules are overfilled to ensure a minimum 1 mL volume can be transferred when using a 1 mL Class A volumetric pipette. We advise laboratories to quantitatively transfer desired volumes of this standard using established good laboratory practices to dilute to the desired concentration.

Component	Solution Purity	Certified Concentration
Amitriptyline Hydrochloride Amitriptyline Hydrochloride	99.8%	1.000 ± 0.006 mg/mL (as free base)

mg/mL (as base)

- Uncertainty of the concentration is expressed as an expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k = 2 and has been calculated by statistical analysis of our production system and incorporates uncertainty of the purity factor, material density, and balance and weighing technique.
- This standard is prepared gravimetrically and mass results are reported on the conventional basis for weighing in air. Concentration is calculated based on: the actual measured mass; Purity Factor of the analyte(s); measured mass of the solution; and the density of the pure diluent at 20°C.
- Concentration is corrected for chromatographic purity, residual water, residual solvents and residual inorganics.

Solution Standard Verification and Homogeneity

Standard Solution	Lot Number	Verified Concentration (mg/mL) Actual Results	Verified Concentration (mg/mL) Acceptance Criteria	%RSD - Homogeneity Actual Results	%RSD - Homogeneity Acceptance Criteria
New Lot	FN102912-01	1.002	± 3%	0.9	≤ 3%
Previous Lot	FN062910-01	1.001	± 3%		

- Concentration is verified through multiple analyses and is calculated as the average of multiple analyses compared to an independently prepared calibration solution.
- Homogeneity of the New Lot is ensured through rigorous production process controls statistically analyzed to evaluate risk and verified by analysis. The % RSD of samples pulled from across the lot demonstrates homogeneity of the New Lot.

Traceability

- Gravimetrically prepared using qualified balances calibrated semi-annually by Mettler Toledo using NIST traceable weights. Calibration verification performed weekly and prior to each use utilizing NIST traceable weights. Each balance has been assigned a minimum weighing by Mettler Toledo taking into consideration the balance and installed environmental conditions to ensure weighing complies with USP tolerances of no more than 0.1% relative error.
- Concentration is verified against an independently prepared calibration solution gravimetrically prepared using balances calibrated to NIST.
- In addition, each neat material utilized has been identified and thoroughly characterized through the use of multiple analytical techniques. Spectral data is provided on subsequent pages of the COA.

Cerilliant certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration/retest date when stored unopened as recommended. Product should be used shortly after opening to avoid concentration changes due to evaporation. Warranty does not apply to ampoules stored after opening.

ACLASS

Lara Sparks, Quality Assurance Director

December 27, 2012
Date

Standard Solution Assay Parameters

Analysis Method:	UV/Vis
Wavelength:	206 nm
Slit Width:	1.0 nm
Response:	0.5 s

Calibration Curve

Calibration Curve:	Linear Regression
Number of Points:	4
Linearity (r) :	1.000

Neat Material Data

Compound Name:	Amitriptyline Hydrochloride
Compound Lot:	PN110612-01
Chemical Formula:	$C_{20}H_{23}N \cdot HCl$
CAS Number:	549-18-8
Molecular Weight:	313.86

Neat Material Characterization Summary

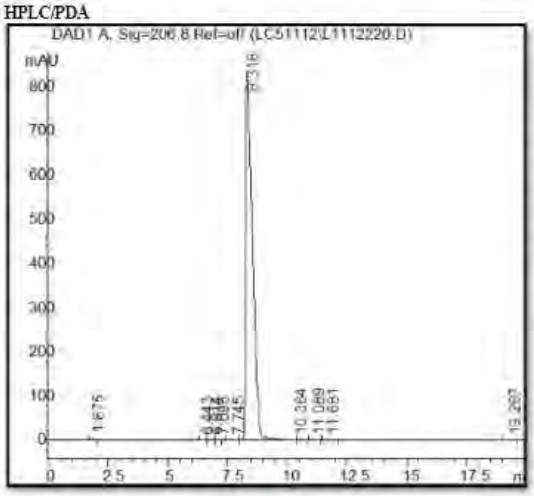
Analytical Test	Method	Results
Primary Chromatographic Purity by HPLC/PDA Analysis	SP10-0102	99.8%
Secondary Purity Analysis by GC/FID	SP10-0101	99.8%
Identity by LC/MS Analysis	SP10-0107	Consistent with Structure
Identity by ^{1}H-NMR Analysis	USP <761>, SP10-0116	Consistent with Structure
Residual Solvent Analysis by GC/FID Headspace	AM1087 [1]	0.01%
Residual Water Analysis by Karl Fischer Coulometry	AM1346 [1]	ND
Inorganic Content by Microash Analysis	SP10-0135	<0.2%
Purity Factor		99.81%

- Primary purity is calculated as the average of two independently performed analyses utilizing two different methods. Acceptance criteria requires the purity values to be within 0.5% of each other.
- The primary chromatographic purity value is used to calculate the Purity Factor, A.
- secondary chromatographic purity method is utilized as a control.
- Purity Factor = [(100 - wt% residual solvent - wt% residual water - wt% residual inorganics) x Chromatographic Purity/100].
- Purity factor does not include adjustment for chiral and/or isotopic purity. Validated analytical method.

[1]

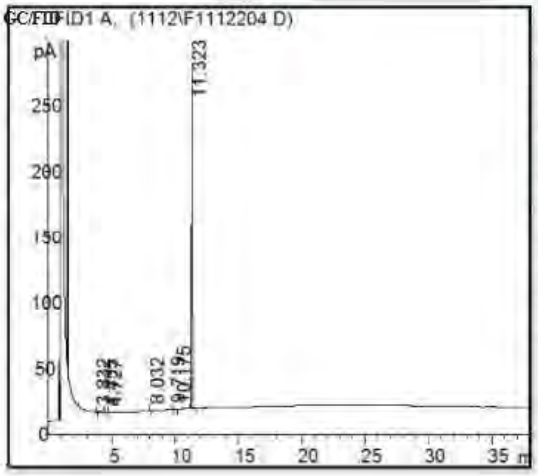
Spectral and Physical Data

HPLC/PDA



Column:	Synergi Polar RP 4µ, 4.6 x 250 mm
Mobile Phase:	Acetonitrile::10mm KH_2PO_4 (70::30)
Flow Rate:	1.0 mL/min
Wavelength:	206 nm
Data File Name:	S:\HPLC\HPLC5\2012\LC51112\L1112220.D
Operator:	ERW
Instrument:	LC#5
Sample Name:	FN110612-01
Method File:	RMA032-2.M
Acquired:	November 12, 2012 4:23 PM

Peak#	Ret Time	Area	Height	Area %
1	1.88	12.11	3.63	0.07
2	6.44	2.07	0.22	0.01
3	6.81	2.03	0.28	0.01
4	7.10	1.08	0.15	0.01
5	7.75	1.28	0.09	0.01
6	8.32	16164.20	835.89	99.85
7	10.36	4.18	0.29	0.03
8	11.09	1.4040	0.1212	0.01
01				
9	11.68	1.58	0.10	0.01
10	19.29	1.78	0.09	0.01



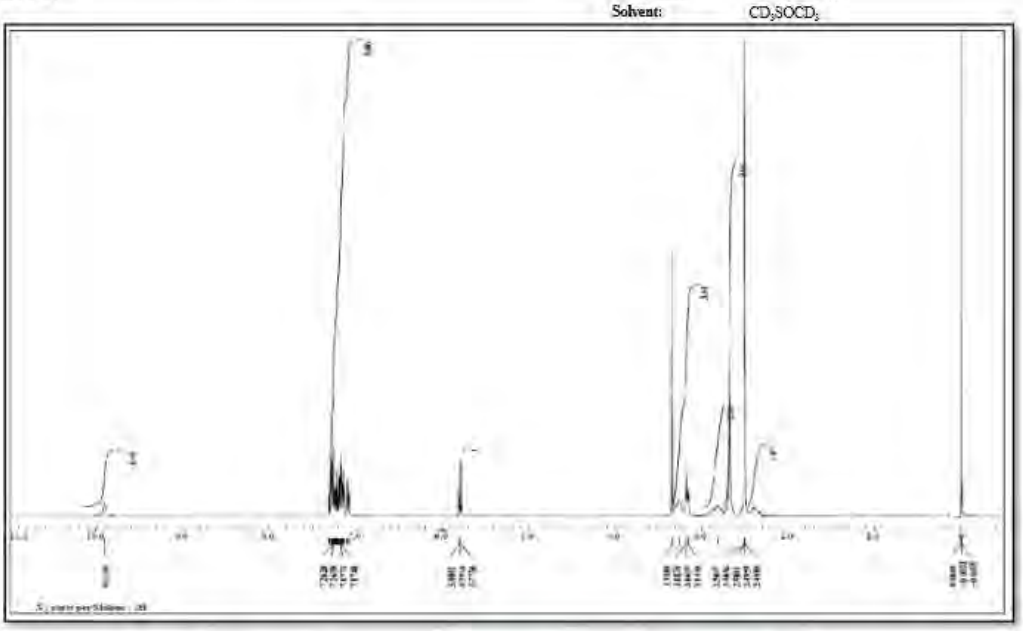
Column:	DB-5ms, 30 m x 0.53 mm ID, 1.5 µm film thickness
Temp Program:	40°C to 200°C at 40°C/min 200°C to 280°C at 5°C/min (hold 18 min)
Injector Temp:	Cool-on-Column
Detector Temp:	325 °C
Data File Name:	S:\GC\GC6\2012\1112\F1112204.D
Operator:	RPC
Instrument:	GC#6
Sample Name:	FN110612-01
Method File:	B01E.M
Acquired:	November 12, 2012 3:36 PM

Peak#	Ret Time	Area	Height	Area %
1	3.83	0.53	0.50	0.05
2	4.44	0.37	0.28	0.03
3	4.73	0.28	0.21	0.03
4	8.03	0.44	0.11	0.04
5	9.72	0.22	0.07	0.02
6	10.18	0.36	0.05	0.03
7	11.32	1073.72	257.55	99.79

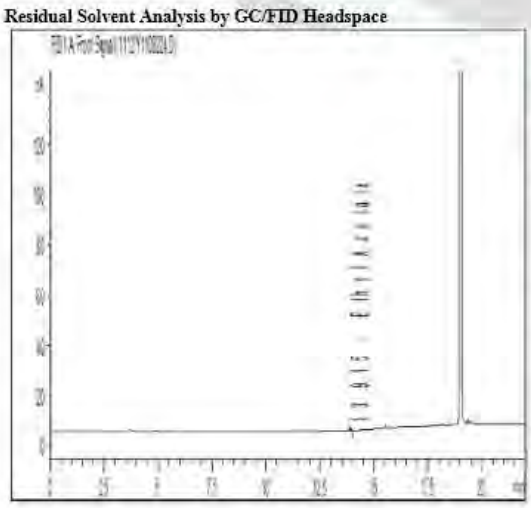
Spectral and Physical Data (cont.)

^{1}H NMR

Instrument: JEOL ECS 400
Solvent: CD_3SOCD_3



Residual Solvent Analysis by GC/FID Headspace

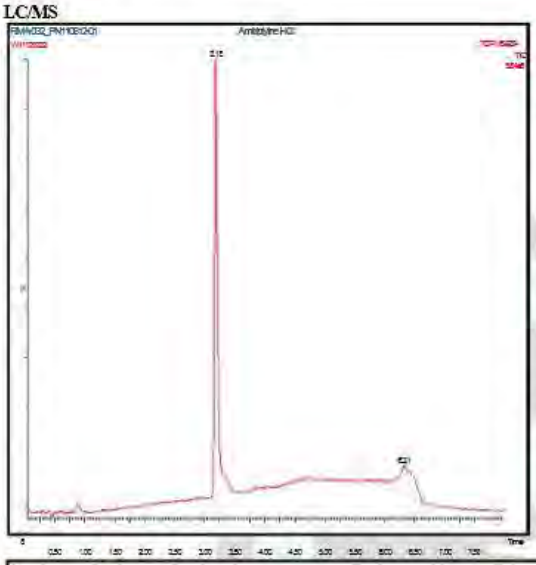


Column:	DB-ALC1 30 m x 0.53 mm, 3 µm film thickness
Temp Program:	40°C (12 min) to 220°C at 40°C/min (5.5 min)
Carrier Gas:	Helium
Flow Rate:	2.0 mL/min
Detector Heater Temp:	250 °C
Injector:	Headspace Sampler
HS Oven Temp:	60 °C
Vial Equilibration:	10 minutes
Data File Name:	S:\GC\GC-HS11\2012\1112\Y1109224.D
Operator:	BD
Instrument:	GC#11
Sample Name:	PN110612-01
Acquired:	November 10, 2012 1:14 AM

Peak	Compound	Area	Weight %
1	Ethyl acetate	2.33955	0.01
2	NMP	NA	NA
Total	**0.01**		

Spectral and Physical Data (cont.)

LC/MS

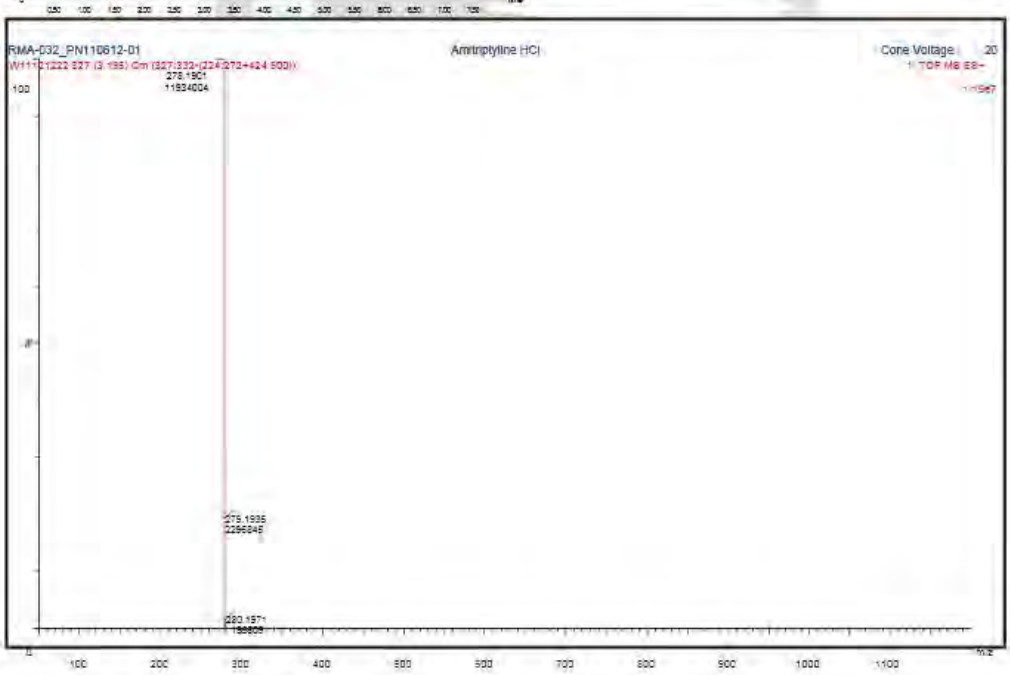


Column:	Zorbax Eclipse Plus C_{18}, Rapid Resolution
Mobile Phase:	A: 0.1% Formic acid in water B: Acetonitrile

Gradient Program:

Time (mins)	%A	%B
0.0	90	10
0.5	90	10
4.0	50	50
5.8	50	50
6.0	90	10
8.0	90	10

Flow Rate:	0.4 mL/min
Scan Range:	50 - 1200amu
Ionization:	Electrospray, Positive Ion
Data File Name:	W11121222
Operator:	EW
Instrument:	LC/MS
Sample Name:	FN110612-01
Method File:	19-55C3
Acquired: Acquired:	November 21, 2012 12:00 AM November 12:00



Stability

Short Term Stability *: A summary of accelerated stability findings for a related product (Amitriptyline-D_3 HCl) is listed below.*		
Storage Condition	**Mean Kinetic Temperature (MKT)**	**Time Period**
Freezer	-15°C	No decrease in purity was noted after one week.
Refrigerator	4°C	
Room Temperature	21°C	
40°C	40°C	
Transport/Shipping *: Stability data supports transport of this product at ambient conditions.*		
Short Term Storage: *Stability data supports short term storage up to 1 year at Refrigerate conditions.*		

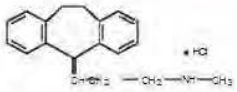
Cerilliant
Analytical Reference Standards
a SIGMA-ALDRICH company

Certificate of Analysis

ISO GUIDE 34
ISO/IEC 17025
ISO 13485
ISO 9001
GMP/GLP

Nortriptyline HCl

3-(10,11-Dihydro-5H-dibenzo[a,d]-cycloheptien-5-ylidene)-methyl-1-propanamine hydrochloride



Catalog Number:	N-907
Solution Lot:	FN110711-03
Expiration Date:	November 2016
Solvent:	Methanol
Volume per Ampule:	Not less than 1 mL
Storage:	Store unopened in freezer.
Shipping:	Ambient. See Stability Section.
Intended Use:	For R&D/ analytical purposes only. Not suitable for human or animal consumption.
Safety:	Flammable, Poison

- Expiration Date has been established through real time stability studies.
- Ampules are overfilled to ensure a minimum 1 mL volume fill. We advise laboratories to use measured volumes of this standard solution before diluting to the desired concentration.

Component	Solution Purity	Certified Concentration
Nortriptyline HCl	99.8%	1.000 ± 0.006 mg/mL (as free base)

- Uncertainty of the concentration is expressed as an expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k = 2 and has been calculated by statistical analysis of our production system and incorporates uncertainty of the purity factor, material density, and balance and weighing technique.
- This standard is prepared gravimetrically and mass results are reported on the conventional basis for weighing in air. Concentration is calculated based on the actual measured mass, Purity Factor of the analyte(s), measured mass of the solution, and the density of the pure diluent at 20C °
- Concentration is corrected for chromatographic purity, residual water, residual solvents and residual inorganics.

Solution Standard Verification and Homogeneity

Standard Solution	Lot Number	Verified Concentration (mg/mL) Actual Results	Verified Concentration (mg/mL) Acceptance Criteria	%RSD - Homogeneity Actual Results	%RSD - Homogeneity Acceptance Criteria
New Lot	FN110711-03	0.992	±3%	0.8	≤3%
Previous Lot	FN010909-01	0.999	±3%		

- Concentration is verified through multiple analyses and is calculated as the average of multiple analyses compared to an independently prepared calibration solution.
- Homogeneity of the New Lot is ensured through rigorous production process controls statistically analyzed to evaluate risk and verified by analysis. The %RSD of samples pulled from across the lot demonstrate homogeneity of the New Lot.

Traceability

- Gravimetrically prepared using qualified balances calibrated semi-annually by Mettler Toledo using NIST traceable weights. Calibration verification performed weekly and prior to each use utilizing NIST traceable weights. Each balance has been assigned a minimum weighing by Mettler Toledo taking into consideration the balance and installed environmental conditions to ensure weighing complies with USP tolerances of no more than 0.1% relative error.
- Concentration is verified against an independently prepared calibration solution gravimetrically prepared using balances calibrated to NIST.
- In addition, each neat material utilized has been identified and thoroughly characterized through the use of multiple analytical techniques. Spectral data is provided on subsequent pages of the COA.

Cerilliant certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration/retest date when stored unopened as recommended. Product should be used shortly after opening to avoid concentration changes due to evaporation. Warranty does not apply to ampoules stored after opening.

ACLASS

Lara Sparks, Quality Assurance Director

March 16, 2012
Date

Standard Solution Assay Parameters

Analysis Method:	UV/Vis
Wavelength:	270 nm
Slit Width:	1.0 nm
Response:	0.5 s

Calibration Curve

Calibration Curve:	Linear Regression
Number of Points:	4
Linearity (r) :	1.000

Neat Material Data

Compound Name:	Nortriptyline HCl	**Chemical Formula:**	$C_{19}H_{21}N$·HCl
Compound Lot:	PN032910-43	**CAS Number:**	894-71-3
		Molecular Weight:	299.84

Neat Material Characterization Summary

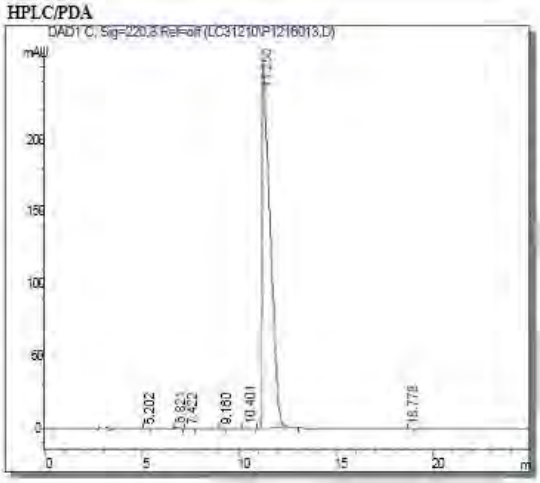
Analytical Test	Method	Results
Primary Chromatographic Purity by HPLC/PDA Analysis	SP10-0102	99.9%
Secondary Chromatographic Purity by GC/FID Analysis	SP10-0101	99.9%
Identity by LC/MS Analysis yy y	SP10-0107	Consistent with Structure
Identity by ^{1}H-NMR Analysis	USP <761>, SP10-0116	Consistent with Structure
Residual Solvent Analysis by GC/FID Headspace	AM1087 [1]	None Detected
Residual Water Analysis by Karl Fischer Coulometry	USP <921>, SP10-0103	Not Detected
Inorganic Content by Microash Analysis	SP10-0135	<0.2%
Purity Factor		99.85%

- Primary purity is calculated as the average of two independently performed analyses utilizing two different methods. Acceptance criteria requires the purity values to be within 0.5% of each other.
- The primary chromatographic purity value is used to calculate the Purity Factor. A
- secondary chromatographic purity method is utilized as a control.
- Purity Factor = [(100 - wt% residual solvent - wt% residual water - wt% residual inorganics) x Chromatographic Purity/100].
- Purity factor does not include adjustment for chiral and/or isotopic purity.

[1] Validated analytical method.

Spectral and Physical Data

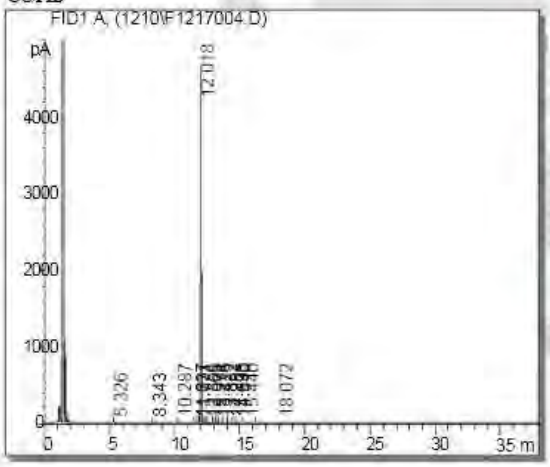
HPLC/PDA



Column:	Gemini C18 5µ, 4.6 x 250 mm
Mobile Phase:	Acetonitrile: Water::0.1% H_3PO_4 (30::35::35)
Flow Rate:	1.0 mL/min
Wavelength:	220 nm
Data File Name:	S:\HPLC\HPLC3\2010\LC31210\P1216013.D
Operator:	GLP
Instrument:	LC#3
Sample Name:	PN032910-43
Method File:	RMN-033.M
Acquired:	December 16, 2010 8:28 PM

Peak#	Ret Time	Area	Height	Area %
1	5.20	0.73	0.07	0.01
2	6.82	5.41	0.57	0.08
3	7.42	1.39	0.09	0.02
4	9.16	0.66	0.06	0.01
5	10.40	3.34	0.18	0.05
6	11.25	7161.55	251.23	99.83
7	18.78	0.71	0.05	0.01

GC/FID

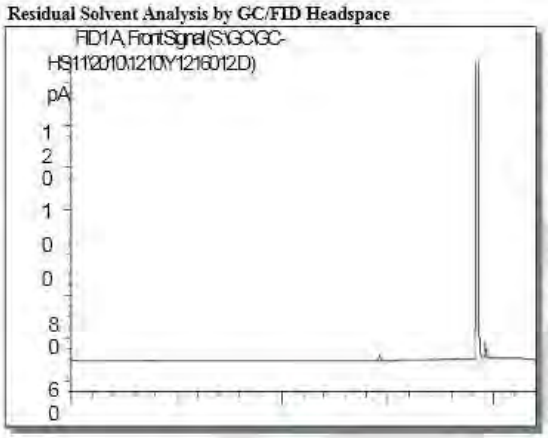


Column:	DB-5ms, 30m x 0.53mm ID, 1.5 µm film thickness
Temp Program:	40°C to 200°C at 40°C/min 200°C to 280°C at 5°C/min (hold 18 min)
Injector Temp:	Cool-on-column
Detector Temp:	325°C
Data File Name:	S:\GC\GC6\2010\1210\F1217004.D
Operator:	PT
Instrument:	GC#6
Sample Name:	PN032910-43
Method File:	AM1043.M
Acquired:	December 17, 2010 4:36 PM

Peak#	Ret Time	Area	Height	Area %
1	5.33	0.46	0.24	0.00
2	8.34	0.91	0.27	0.00
3	10.29	0.03	0.15	0.00
4	11.64	0.85	0.20	0.00
5	11.77	0.73	0.15	0.00
6	12.02	19442.60	4577.53	99.86
7	12.36	4.79	0.75	0.02
8	12.57	8.03	1.68	0.04
9	12.97	1.24	0.16	0.01
10	13.22	1.73	0.34	0.01
11	13.43	1.14	0.20	0.01
12	13.83	0.97	0.16	0.00
13	14.25	1.27	0.18	0.01
14	14.70	0.69	0.10	0.00
15	14.84	0.81	0.16	0.00
16	15.44	0.90	0.11	0.00
17	18.07	2.18	0.14	0.01

Spectral and Physical Data (cont.)

Residual Solvent Analysis by GC/FID Headspace



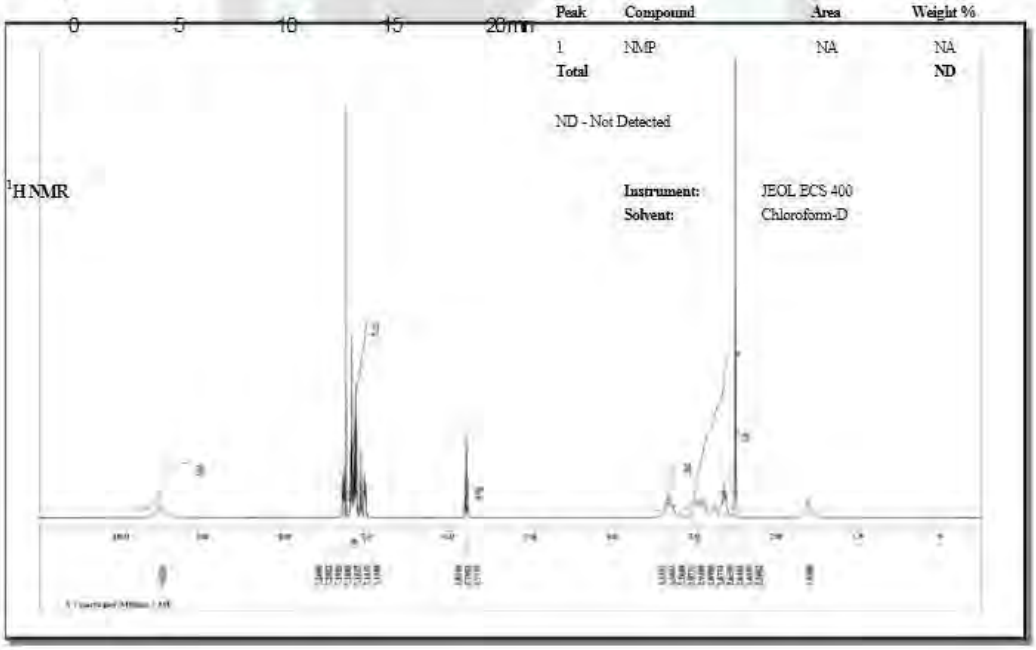
Column:	DB-ALC1 30 m x 0.53 mm, 3 um film thickness
Temp Program:	40°C (12 min) to 220°C at 40°C/min (5.5 min)
Carrier Gas:	Helium
Flow Rate:	2.0 mL/min
Detector Heater Temp:	250°C
Injector:	Headspace Sampler
HS Oven Temp:	60°C
Vial Equilibration:	10 minutes
Data File Name:	S:\GC\GC-HS11\2010\1210\Y1216012.D
Operator:	BD
Instrument:	GC#11
Sample Name:	PN032910-43
Acquired:	December 17, 2010 8:58 PM

Peak	Compound	Area	Weight %
1	NMP	NA	NA
Total			**ND**

ND - Not Detected

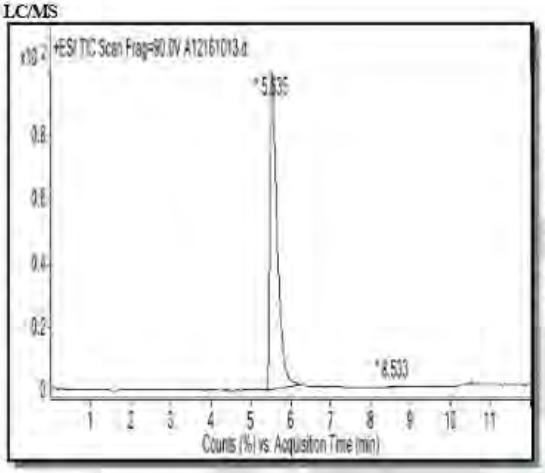
^{1}H NMR

Instrument:	JEOL ECS 400
Solvent:	Chloroform-D

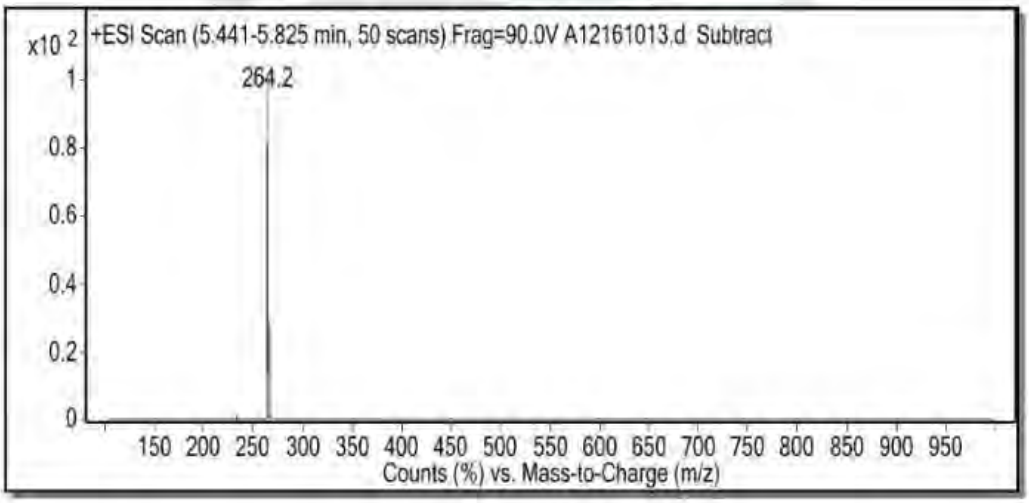


Spectral and Physical Data (cont.)

LC/MS



Column:	Luna 3μ C18 (2), 2.0 x 100 mm
Mobile Phase:	Acetonitrile::0.1% Formic acid in Water (10::90 to 50::50 at 6 mins, hold 9 mins)
Flow Rate:	0.3 mL/min
Scan Range:	100-1000 amu
Ionization:	Electrospray, Positive Ion
Data File Name:	A12161013.d
Operator:	HJ
Instrument:	LC/MS/MS
Sample Name:	PN032910-43
Method File:	28-82C2P.m
Acquired:	December 16, 2010 5:29 PM



Stability

Short Term Stability *: A summary of accelerated stability findings for this product is listed below.*

Storage Condition	Mean Kinetic Temperature (MKT)	Time Period
Freezer	-15°C	No decrease in purity was noted after one week.
Refrigerator	4°C	
Room Temperature	21°C	
40°C	40°C	

Transport/Shipping *: Stability data supports transport of this product at ambient conditions.*

Short Term Storage: *Stability data supports short term storage up to 1 year at Refrigerate conditions.*

Cerilliant
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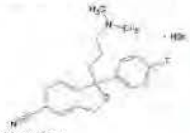
Certified Reference Material - Certificate of Analysis

Citalopram, Primary Standard

ISO GUIDE 34
ISO/IEC 17025
ISO 13485
ISO 9001
GMP/QSP



Catalog Number: C-057
Lot: FN100410-03 October
Expiration: 2014
Description: Citalopram HBr in Methanol
Nominal concentration is adjusted for HBr content.
Packaging: Solution in 2 mL amber USP Type I glass ampoule containing not less than 1 mL of certified solution.
Storage: Store unopened in freezer (-10 °C to -25 °C). See Stability Section for short-term storage.
Shipping: Ambient. See Stability Section.
Intended Use: This Certified Reference Material is suitable for the *in vitro* identification, calibration, and quantification of the analyte(s) in analytical and R&D applications. Not suitable for human or animal consumption.
Instructions for Use: Users should quantitatively transfer desired volume using established good laboratory practices to spike into matrix or to dilute to the desired concentration. Each ampoule is intended for one-time use.
Safety: **Flammable, Poison. See Safety Data Sheet**

- Expiration Date has been established through real time stability studies.
- Ampoules are overfilled to ensure a minimum 1 mL volume can be transferred when using a 1 mL Class A volumetric pipette.

using mL

Analyte	Certified Concentration Value
Citalopram	100.0 ± 0.5 µg/mL

- Uncertainty of the concentration is expressed as an expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using
- a coverage factor of k = 2 and has been calculated by statistical analysis of our production system and incorporates uncertainty of the mass balance purity factor, material density, balance, and weighing technique.
- This standard is prepared gravimetrically and mass results are reported on the conventional basis for weighing in air. Nominal concentration is calculated based on: the
- actual measured mass, Mass Balance Purity Factor of the analyte(s), measured mass of the solution; and the density of the pure diluent at 20 °C.
- Concentration is corrected for chromatographic purity, residual water, residual solvents and residual inorganics. Nominal concentration is adjusted for HCl content.
No adjustment required before use.
- Additional certification documentation available upon request.

Metrological Traceability:

- This standard has been prepared and certified under the ISO Guide 34, ISO/IEC 17025, ISO 9001 and ISO 13485 standards. This standard meets the requirements of a Certified Reference Material and a Primary Standard as defined by ISO and is traceable to the SI and higher order standards through an unbroken chain of comparisons.
- This standard has been gravimetrically prepared using balances that have been fully qualified and calibrated to ISO 17025 requirements. All calibrations utilize NIST traceable weights which are calibrated externally by a qualified ISO 17025 accredited calibration laboratory to NIST standards. Qualification of each balance includes the assignment of a minimum weighing by a qualified and ISO 17025 accredited calibration vendor taking into consideration the balance and installed environmental conditions to ensure compliance with USP tolerances of NMT 0.1% relative uncertainty. Balance calibration adjustments are performed weekly utilizing the balance's internal adjustment mechanism. Calibration verifications are performed pre-use. Weigh tapes from the calibration verification are included in the production batch record for this standard. Production data package available upon request.
- Fill volume is gravimetrically verified throughout the dispensing process using qualified and calibrated balances.
- Concentration is verified against an independently prepared calibration solution gravimetrically prepared.
- Each raw material utilized has been identified and thoroughly characterized through the use of multiple analytical techniques. Spectral data is provided on subsequent pages of this COA. The density and material Mass Balance Purity Factor is traceable to the SI and higher order reference standards through mass measurement and instrument qualification and calibration.

Cerilliant certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration/retest date when stored unopened as recommended. Product should be used shortly after opening to avoid concentration changes due to evaporation. Warranty does not apply to ampoules stored after opening.

ACLASS

Darron Ellsworth, Quality Assurance Manager — July 24, 2013 Date

Solution Standard Verification

Concentration accuracy and within- and between-bottle homogeneity are analytically verified against an independently prepared calibration solution and to the prior lot.

Solution standard verification demonstrates confirmation that the specified requirements for the Primary Standard have been fulfilled and validated under ISO 13485.

Standard Solution Assay Parameters		Calibration Curve	
Analysis Method:	UV/Vis	**Calibration Curve:**	Linear Regression
Wavelength:	238 nm	**Number of Points:**	4
Slit Width:	1.0 nm	**Linearity (r) :**	1.000
Response:	0.5 s		

Standard Solution	Lot Number	Verified Concentration (µg/mL) Actual Results	Verified Concentration (µg/mL) Acceptance Criteria	%RSD - Homogeneity Actual Results	%RSD - Homogeneity Acceptance Criteria
New Lot	FN100410-03	98.9	±3%	0.2	≤3%
Previous Lot	FN092408-02	100.3	±3%		

- Concentration is verified through multiple analyses and is calculated as the average of multiple analyses compared to an independently prepared calibration solution.
- Within-sample and between-sample homogeneity of the New Lot is ensured through rigorous production process controls statistically analyzed to evaluate risk and verified by analysis. Multiple samples pulled from across the lot using a random stratified sampling plan were analyzed to verify homogeneity. % RSD results shown above for the New Lot demonstrate ampoule-to-ampoule homogeneity.

Analyte Certification Mass Balance Purity Factor
Analyte Certification - Mass Balance Purity Factor

Each analyte is thoroughly identified and characterized using an orthogonal approach. A mass balance purity factor is assigned incorporating chromatographic purity and residual impurities. The mass balance purity factor and salt adjustment are utilized to calculate the weighing adjustment necessary to ensure accuracy of the solution standard concentration.

Material	Lot Number	CAS Number	Chemical Formula	Molecular Weight (salt)	Molecular Weight (base)	Salt Adjustment
Citalopram HBr	FN072308-01	59729-32-7	$C_{20}H_{21}FN_2O \cdot HBr$	405.31	324.39	1.249

Material Characterization Summary

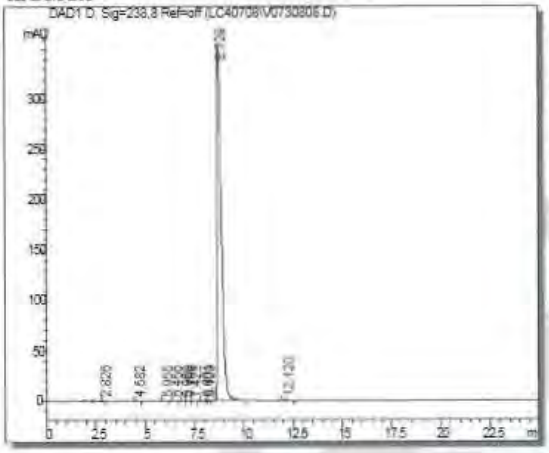
Analytical Test	Method	Results
Primary Chromatographic Purity by HPLC/PDA Analysis	SP10-0102	99.7%
Secondary Purity Analysis by Thin Layer Chromatography	SP10-0106	Single Spot, Rf = 0.43
Identity by LC/MS Analysis	SP10-0107	Consistent with Structure
Identity by ^{1}H-NMR Analysis	USP <761>, SP10-0116	Consistent with Structure
Residual Solvent Analysis by GC/FID Headspace	AM1087 [1]	0.09%
Residual Water Analysis by Karl Fischer Coulometry	USP <921>, SP10-0103	0.17%
Inorganic Content by Microash Analysis	Outsourced	< 0.1%
Mass Balance Purity Factor	99.47%	

- The primary chromatographic purity is calculated as the average of two independently performed analyses utilizing two different methods. Acceptance criteria requires the purity values to be within 0.5% of each other.
- The primary chromatographic purity value is used to calculate the Mass Balance Purity Factor. A
- secondary chromatographic purity method is utilized as a control.
- Mass Balance Purity Factor = [(100 - wt% residual solvent - wt% residual water - wt% residual inorganics) x Chromatographic Purity/100].
- Mass Balance Purity Factor does not include adjustment for chiral and/or isotopic purity.

[1] Validated analytical method

Spectral and Physical Data

HPLC/PDA

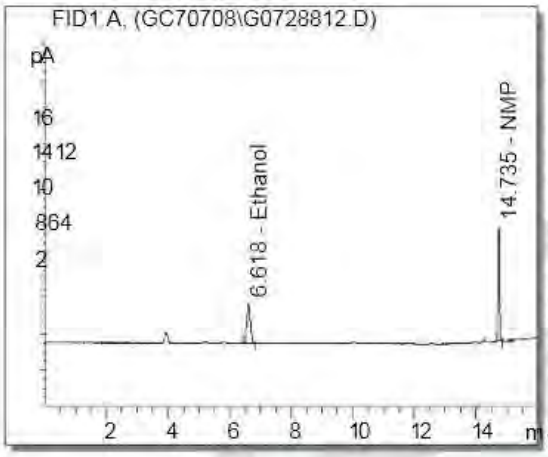


Column:	Synergi Polar RP, 4.6 x 250 mm
Mobile Phase:	Acetonitrile::10 mM Potassium phosphate buffer (75::25)
Flow Rate:	1.0 mL/min
Wavelength:	238 nm
Data File Name:	C:\HPCHEM\1\DATA\LC40708\V0730808.D
Operator:	TNT
Instrument:	LC#4
Sample Name:	PN072308-01
Method File:	RMC062.M
Acquired:	July 30, 2008 2:17 PM

Peak#	Ret Time	Area	Height	Area %
1	2.83	1.36	0.33	0.02
2	4.58	1.44	0.20	0.05
3	5.96	0.97	0.11	0.02
4	6.46	0.93	0.12	0.02
5	6.97	0.70	0.09	0.01
6	7.16	1.56	0.17	0.02
7	7.42 7.42	0.92 0.92	0.10 0.10	0.02 0.02
8	8.00	4.42	0.49	0.08
	8.13	1.97	0.30	0.04
10	8.71	5449.70	353.50	99.67
11	12.12	4.08	0.26	0.07

Spectral and Physical Data (cont.)

Residual Solvent Analysis by GC/FID Headspace

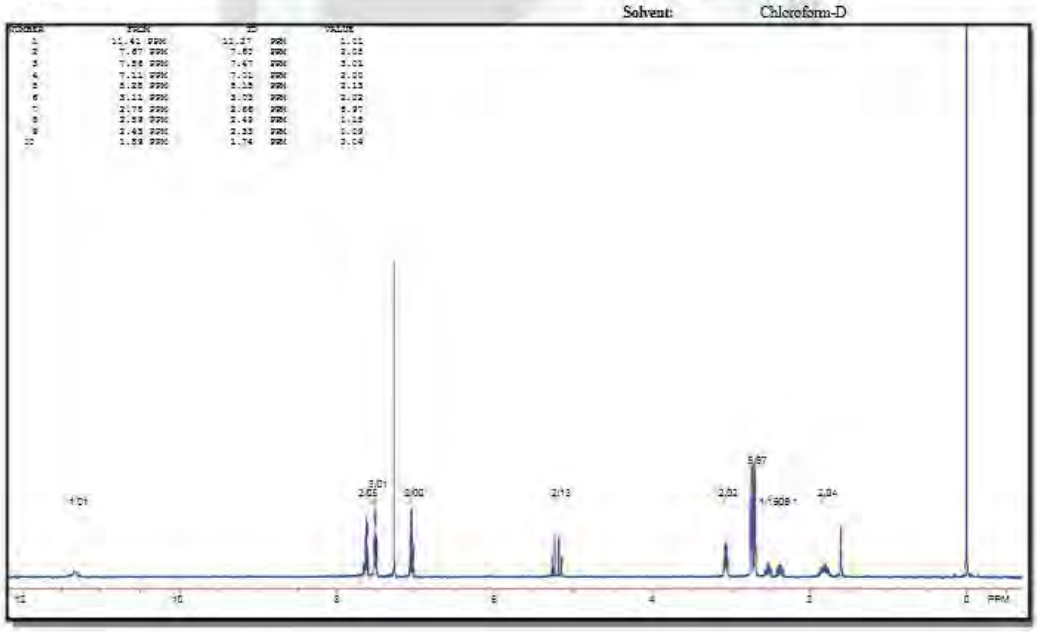


Column:	DB-ALC1 30 m x 0.53 mm, 3 µm film thickness
Temp Program:	40°C (12 min) to 220°C at 40°C/min (5.5 min)
Carrier Gas:	Helium
Flow Rate:	2.0 mL/min
Detector Temp:	250°C
Injector:	Headspace Sampler
Injector Temp:	200°C
HS Oven Temp:	200°C 1.0
Injection Volume:	mL
Incubation Time:	10 minutes
Data File Name:	C:\HPCHEM\1\DATA\GC70708\G0728812.D
Operator:	KRS
Instrument:	GC#7
Sample Name:	PN072308-01
Acquired:	July 29, 2008 10:12 AM

Peak	Compound	Area	Weight %
1	Ethanol	16.23812	0.09
2	NMP	NA	NA
Total			**0.09**

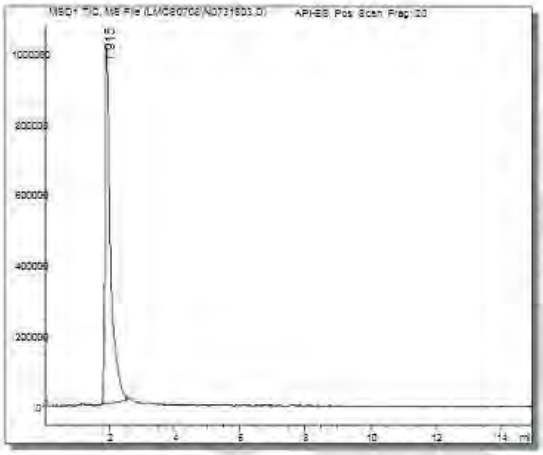
^{1}H NMR

Instrument: Bruker DRX 400
Solvent: Chloroform-D

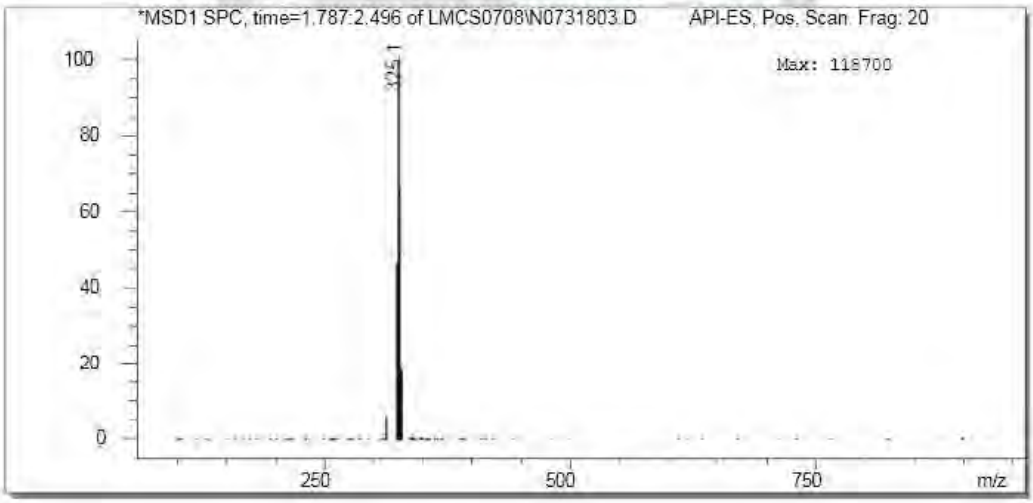


Spectral and Physical Data (cont.)

LC/MS



Column:	Luna C_{18} 2.0 x 100 mm
Mobile Phase:	Acetonitrile: 10 mM Ammonium acetate buffer (75:25)
Flow Rate:	0.2 mL/min
Scan Range:	100-1000 amu
Ionization:	Electrospray, Positive Ion
Data File Name:	C:\HPCHEM\1\DATA\LCMS0708\N0731803.D
Operator:	MAM
Instrument:	LCMS1
Sample Name:	FN072308-01
Method File:	RMC062.M
Acquired:	July 31, 2008 11:11 AM



Stability

Short term stability studies have been performed under accelerated conditions for periods up to one week. Short term data is utilized to predict long term stability and to support transport conditions and normal laboratory use. Real-time stability studies are performed at the recommended storage conditions over the life of the product.

***Short Term Stability** : A summary of accelerated stability findings for this product is listed below.*		
Storage Condition	**Mean Kinetic Temperature (MKT)**	**Time Period/Result**
Freezer	-15°C	No decrease in purity was noted after one week
Refrigerator	4°C	
Room Temperature	21°C	
40°C	40°C	
***Transport/Shipping** : Stability data supports transport of this product at ambient conditions.*		
***Short Term Storage:** Stability data supports short term storage up to 12 months at refrigerator conditions.*		
***Long Term Stability:** Long term stability has been assessed for freezer storage (-10 °C to -25 °C) conditions. Stability of a minimum of 52 months has been established through real-time stability studies.*		

COA Revision History

Revision No.	Date	Reason for Revision
00	10/24/2010	Initial version.
01	1/20/2012	Revised Storage condition from "Refrigerate or freeze" to "Store in freezer."
02	7/24/2013	General rewrite. Added Stability section.

Certificate of Analysis

ISO GUIDE 34
ISO/IEC 17025
ISO 13485
ISO 9001
GMP/GLP

Benzodiazepine Multi-Component Mixture-8

Catalog Number: B-033
Solution Lot: FE012413-02 January
Expiration Date: 2018
Solvent: Acetonitrile (LC-MS Chromasolv®)
Volume per Ampule: Not less than 1 mL
Storage: Store in freezer
Intended Use: For R&D analytical purposes only. Not suitable for human or animal consumption.
Regulatory: USDEA Exempt | Canadian TK # 61-466 **Safety:** Flammable, Poison

- Expiration Date has been established through real time stability studies.
- Ampules are overfilled to ensure a minimum 1 mL volume can be transferred when using a 1 mL Class A volumetric pipette. We advise laboratories to quantitatively transfer desired volumes of this standard using established good laboratory practices to dilute to the desired concentration.

Component	Chromatographic Purity	Concentration
Alprazolam	99.72%	250.0 1.6 / L 250 0
Al I	99.72%	± 1.6 µg/mL
Clonazepam	99.79%	249.9 ± 1.6 µg/mL
Diazepam	99.98%	249.9 ± 1.6 µg/mL
Flunitrazepam	99.90%	250.0 ± 1.6 µg/mL
Lorazepam	99.74%	250.0 ± 1.6 µg/mL
Nitrazepam	99.91%	250.0 ± 1.6 µg/mL
Oxazepam	99.75%	250.0 ± 1.6 µg/mL
Temazepam	99.98%	250.0 ± 1.6 µg/mL

- Uncertainty of the concentration is expressed as an expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k = 2 and has been calculated by statistical analysis of our production system and incorporates uncertainty of the purity factor, material density, and balance and weighing technique.
- This standard is prepared gravimetrically and mass results are reported on the conventional basis for weighing in air. Concentration is calculated based on: the actual measured mass; Purity Factor of the analyte(s); measured mass of the solution; and the density of the pure diluent at 20 °C.
- Concentration is corrected for chromatographic purity, residual water, residual solvents and residual inorganics.

Traceability

- Gravimetrically prepared using qualified balances calibrated semi-annually by Mettler Toledo using NIST traceable weights. Calibration verification performed weekly and prior to each use utilizing NIST traceable weights. Each balance has been assigned a minimum weighing by Mettler Toledo taking into consideration the balance and installed environmental conditions to ensure weighing complies with USP tolerances of no more than 0.1% relative error.
- Concentration is verified against an independently prepared 4-point calibration curve gravimetrically prepared using balances calibrated to NIST.
- In addition, each neat material utilized has been identified and thoroughly characterized through the use of multiple analytical techniques. Spectral data is provided on subsequent pages of the COA.

Cerilliant certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration/retest date when stored unopened as recommended. Product should be used shortly after opening to avoid concentration changes due to evaporation. Warranty does not apply to ampoules stored after opening.

Lara Sparks, Quality Assurance Director — *February 12, 2013* Date

Standard Solution Assay Parameters

Analysis Method:	HPLC/UV
Column:	Betasil Phenyl, 4.6 x 150 mm
Mobile Phase:	A: Acetonitrile B: 1 mM Ammonium Acetate Buffer

Gradient Program:

Time (mins)	%A	%B
0	40	60
20	60	40

Flow Rate:	1.0 mL/min
Wavelength:	267 nm

Calibration Curve

Calibration Curve:	Linear Regression
Number of Points:	4
Linearity (r) :	≥0.999

Solution Standard Verification and Homogeneity

Compound	Verified Concentration µg/mL	Acceptance Criteria	%RSD - Homogeneity %	Acceptance Criteria
Alprazolam	253.7	±5%	0.4	≤3%
Clonazepam	250.8	±5%	0.2	≤3%
Diazepam	251.8	±5%	0.2	≤3%
Flunitrazepam	251.3	±5%	0.2	≤3%
Lorazepam	250.0	±5%	0.2	≤3%
Nitrazepam	250.3	±5%	0.7	≤3%
Oxazepam Oxazepam	251.8 251.8	±5% %	0.2 0.2	≤3%
Temazepam	250.6	±5%	0.3	≤3%

- Concentration is verified through multiple analyses and is calculated as the average of multiple analyses compared to an independently prepared calibration curve.
- Homogeneity is ensured through rigorous production process controls statistically analyzed to evaluate risk and verified by analysis. The %RSD of samples pulled from across the lot demonstrate homogeneity.
- Product is compared to prior lot and meets acceptance criteria of ± 5%.

Product Analysis

Neat Material Data

Compound	Lot Number	CAS Number	Chemical Formula	Molecular Weight	Identity Confirmed By
Alprazolam	PC081209-01	28981-97-7	$C_{17}H_{13}ClN_4$	308.77	LC/MS
Clonazepam	PC040710-05	1622-61-3	$C_{15}H_{10}ClN_3O_3$	315.72	LC/MS
Diazepam	PC012210-01	439-14-5	$C_{16}H_{13}ClN_2O$	284.74	LC/MS
Flunitrazepam	PC042810-01	1622-62-4	$C_{16}H_{12}FN_3O_3$	313.29	LC/MS
Lorazepam	PC091508-03	846-49-1	$C_{15}H_{10}Cl_2N_2O_2$	321.16	LC/MS
Nitrazepam	PC040710-22	146-22-5	$C_{15}H_{11}N_3O_3$	281.27	LC/MS
Oxazepam	30901-11	604-75-1	$C_{15}H_{11}ClN_2O_2$	286.72	LC/MS
Temazepam	PC120408-07	846-50-4	$C_{16}H_{13}ClN_2O_2$	300.74	LC/MS

Compound	Chromatographic Purity	Residual Solvent[1]	Residual Water	Residual Inorganics	Purity Factor
Alprazolam	99.72%	None Detected	Not Detected	<0.1%	99.72%
Clonazepam	99.79%	None Detected	Not Detected	<0.2%	99.79%
Diazepam	99.98%	None Detected	0.06%	<0.2%	99.92%
Flunitrazepam	99.90%	None Detected	0.04%	<0.2%	99.86%
Lorazepam	99.74%	0.20%	Not Detected	<0.1%	99.54%
NitNitrazepam 99.87%	99.91% 99.91%	0.04% 0.04%	0.01% 0.01%	<0.2% 0.2%	99.87%
Oxazepam	99.75%	None Detected	0.04%	<0.2%	99.71%
Temazepam	99.98%	1.37%	0.11%	<0.1%	98.50%

- Purity is calculated as the average of two independently performed analyses utilizing two different methods. Acceptance criteria requires the purity values to be within 0.5% of each other. A secondary purity method is used as a control but is not reported in the Certificate of Analysis.
- Purity Factor = [(100 - wt% residual solvent - wt% residual water - wt% residual inorganics) x Chromatographic Purity/100]. Purity factor does not include adjustment for chiral and/or isotopic purity.

[1] Validated analytical method.

RESTEK CERTIFIED REFERENCE MATERIAL

110 Benner Circle
Bellefonte, PA 16823-8812
Tel: (800)356-1688
Fax: (814)353-1309

www.restek.com

Certificate of Analysis

FOR LABORATORY USE ONLY-READ SDS PRIOR TO USE.

This Reference Material is intended for Laboratory Use Only as a standard for the qualitative and/or quantitative determination of the analyte(s) listed.

atalog No.: 36341 **Lot No.:** A095097

escription: Forensic Drug Screen Internal Std Solution #1

Internal Std Solution #1 10µg/mL P&T Methanol 1mL/ampul

ontainer Size: **Pkg Amt:**

piration Date: April 30, 2015 **Storage:** 10°C or colder

CERTIFIED VALUES

Elution Order	Compound		Grav. Conc. (weight/volume)		Expanded Uncertainty (95% C.L.; K=2)		
1	Doxepin-d3 HCL (N-methyl-d3)		10.0	µg/mL	+/- 0.0821	µg/mL	Gravimetric
	CAS# 347840-07-7	(Lot M546P2)			+/- 0.8056	µg/mL	Unstressed
	Purity 88%				+/- 1.4846	µg/mL	Stressed
2	Diazepam-d5		10.0	µg/mL	+/- 0.0818	µg/mL	Gravimetric
	CAS# 65854-76-4	(Lot 1B31)			+/- 0.8030	µg/mL	Unstressed
	Purity 99%				+/- 1.4798	µg/mL	Stressed

Solvent: P&T Methanol
CAS# 67-56-1
Purity 99%

lumn:
n x .25mm x .25um
-5 (cat.#10223)

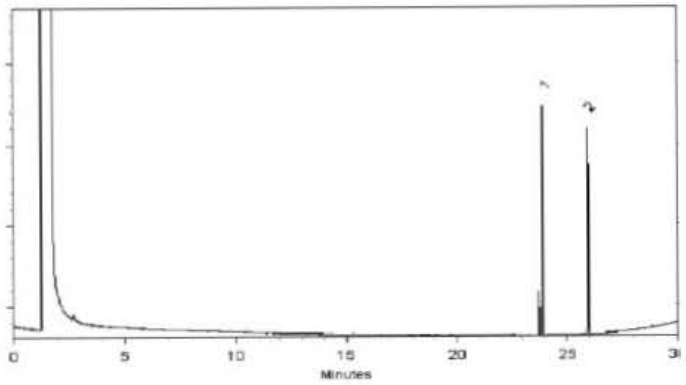
rrier Gas:
lroger--constant pressure 10 psi.

mp. Program:
'C (hold 2 min.) to 330°C
10°C/min. (hold 10 min.)

. Temp:
ºC

t. Temp:
°C

t. Type:



This chromatogram represents a general set of testing conditions chosen to guarantee product quality. For optimal results in your lab, conditions should be adjusted for your specific instrument, method, and application.

F. Joseph Tallon - Mix Technician **Date Mixed:** 29-Apr-2013 **Balance:** B251644995

Jodi E. Breon - QA Analyst **Date Passed:** 08-May-2013

Manufactured under Restek's ISO 9001:2008
Registered Quality System
Certificate #FM 80397

General Certified Reference Material Notes

Expiration Notes:

- Expiration date valid for unopened ampul stored in compliance with the recommended conditions.
- Uncertainty, concentration, and expiration of the CRM are based on the unopened product being stored according to the recommended condition found in the storage field.

Purity Notes:

- Purity and/or chemical identity are determined by one or more of the following techniques: GC/FID, HPLC, GC/μECD, GC/MS, LC/MS, RI, and/or melting point.
- Compounds with a listed purity of less than 99% have been weight corrected to compensate for impurities and/or salts. A correction factor is used to calculate the amount of compound necessary to achieve the desired concentration of the parent compound in solution.
- Purity of isomeric compounds is reported as the sum of the isomers.
- Purity values are rounded to the nearest whole number.

Certified Uncertainty Value Notes:

- The uncertainties are determined in accordance with ISO Guides 34 and 35. The certified combined stressed uncertainty value (includes gravimetric uncertainty, homogeneity between-ampul uncertainty, storage stability uncertainty and shipping stability uncertainty and were combined using the following formula:

$$U_{combined\ stressed} = k\sqrt{U^2_{gravimetric} + U^2_{homogeneity} + U^2_{storage\ stability} + U^2_{shipping\ stability}}$$

  k is a coverage factor of 2, which gives a level of confidence of approximately 95%.
- It is important to note that the shipping stability uncertainty was obtained under temperature extremes for specific time intervals; therefore, the certified combined stressed uncertainty value should only be applied to the product if it was stored at non-standard temperature conditions up to and including 7 days. Contact Restek Technical Service at www.restek.com/Contact-Us for use recommendations if your shipment was in-transit for more than 7 days at non-standard temperature conditions.
- Apply the certified combined unstressed uncertainty value if the product was received under standard shipping conditions. Apply the certified combined stressed uncertainty value if the product was received under non-standard conditions as specified below.

Label Conditions	Standard Conditions	Non-Standard Conditions
25°C Nominal (Room Temperature)	< 60°C	≥ 60°C up to 7 days
10°C or colder (Refrigerate)	< 40°C	≥ 40°C up to 7 days
0°C or colder (Freezer)	< 25°C	≥ 25°C up to 7 days

- Separate (not combined) uncertainty values for gravimetric uncertainty are also displayed on the certificate; if needed, separate homogeneity between-ampul uncertainty, storage stability uncertainty and shipping stability uncertainty values are available by contacting Restek Technical Service at www.restek.com/Contact-Us.
- The packaged amount is the minimum sample size for which uncertainty is valid. The ampules are over-filled to ensure that the minimum packaged amount can be sufficiently transferred.

Manufacturing Notes:

- Concentration is based upon gravimetric preparation using either a balance whose calibration has been verified daily using NIST traceable weights, and/or dilutions with Class A glassware.

Handling Notes:

- Samples should be transferred into deactivated vials for handling and storage. Restek supplies deactivated vials along with most standards packed in 2 mL ampules. Due to space constraints, Restek does not supply vials for larger volume ampules. Restek sells DMDCS for the purpose of glassware deactivation as catalog number 31840, which includes complete instructions. Restek will also deactivate larger volume vials from our inventory as a custom ordered item. Contact your Restek sales or customer service representative for details.
- If any undissolved material is visible inside the ampul, sonicate the unopened ampul until the material is completely dissolved.