

DESIGN AND PROTOTYPE OF AN EXTERNAL
QUALITY ASSURANCE PROGRAM FOR URINE
BICARBONATE

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BY

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This research report is based on my own independent work and has neither as a whole nor in part been, is being or is to be submitted for another degree at any other university. Furthermore, this work has not been published prior to registration for this degree.

Ryan Benjamin

Abstract

This dissertation validates a Beckman-Coulter DxC[®] assay for total bicarbonate in urine and then proceeds to design, prototype and cost an inter-laboratory comparison (ILC) program for the above urine bicarbonate based on the validation. Furthermore, this work serves as a case study for how to establish proficiency testing – and thereby achieve accreditation – for tests without external quality assurance because of analyte instability.

Urine bicarbonate analysis forms part of the diagnostic workup of renal tubular acidosis (RTA). The differential diagnosis of wasting and stunting includes RTA. Given an incidence of wasted and stunted children of 250 000 per annum in South Africa, one should anticipate that at least this many appropriate urine bicarbonates should be requested per annum – thus establishing a need for urine bicarbonate analysis. The provision of urine bicarbonate analysis as an accredited clinical laboratory test requires validation and quality assurance. The absence of proficiency testing for urine bicarbonate diminishes this quality assurance and establishes a problem.

The dissertation also reviews the pathophysiology and diagnosis of RTA. Furthermore, it compares and contrasts the traditional and physicochemical clinical acid-base theories and introduces non-protoncentric acid-base theories.

The study shows that the Beckman-Coulter DxC[®] assay for total bicarbonate in urine has a functional sensitivity of 5 mmol/L . The intra-laboratory coefficient of variation at 10 mmol/L is 18%. The reference range was calculated to be $2 - 4\text{ mmol/L}$.

The ILC program is based on the recovery of total carbon dioxide from urine spiked with sodium bicarbonate. The results are analysed using standard deviation index plots and Youden plots.

For the ILC administering body, the marginal cost of producing the quality control samples and analysing the laboratory results is $R4,64$ per ILC cycle – this is the minimum subscription to the ILC. For the laboratory being evaluated, the marginal cost of the ILC is the subscription plus the marginal cost of analysis. On an arterial blood gas analyser, the marginal cost of analysis is zero and thus the marginal cost of the ILC is $R4,64$. The marginal cost of analysis on the Beckman-Coulter DxC[®] is $R13,00$ per quality control cycle and thus (for the laboratory being evaluated) the minimum marginal cost of the ILC is $R17,64$.

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This document was written in L^AT_EX using MikT_EX, Geany and T_EXnicCenter on both MS windows[®] and linux operating systems. Images were produced using XFig and Gimp. Graphs are from OpenOffice. Statistics was performed on R. This document was referenced with BibT_EX and JabRef.

This dissertation is dedicated to my parents Eric and Marda Benjamin – whose sacrifices (for more than three quarters of a century) are appreciated.



Dissertation notation

All equations are scalar and, where numbered, are identified by chapter name and number where the latter is chronological. Equation references are placed in brackets. Thus the 5th equation in section D1 will be designated (*D1 – 5*). The symbols in all equations are italicised.

Similarly, all numbered reactions are identified by chapter and number but are placed in angled brackets. Thus the 2nd reaction in chapter B would be $\langle B - 2 \rangle$. The symbols within chemical reactions are also italicised.

In Appendix D1, in an attempt to explain various acid-base theories, two vocabularies are constructed. The first vocabulary relates to the description of a phenomenon and the second relates to the explanation of a phenomenon. Consequently, acid-base phenomena are separated into description and explanation.

A patient sample is collected from an individual. The aliquots from a patient sample are specimens. A sample consists of several specimens. Furthermore, a sample represents a collection of specimens from the population such that if a specimen was collected from every individual, within the population, that sample would be the population.

The Road Not Taken

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I marked the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I –
I took the one less traveled by,
And that has made all the difference.

Robert Frost

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List of Abbreviations

$[K^+]_u$	Urine potassium concentration
μ	Mean
CO_2	Carbon dioxide
H^+	Hydrogen ions
HCO_3^-	Bicarbonate
$NaHCO_3$	Sodium bicarbonate
NH_3	Ammonia
NH_4^+	Ammonium
NH_4HCO_3	Ammonium bicarbonate
AAP	Alternate assessment procedures
ABG	Arterial blood gas
AD	Autosomal dominant
AE1	Anion exchanger 1

ANOVA	Analysis of variance
AR	Autosomal recessive
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BE	Base excess
CA	Carbonic anhydrase
Ca	Calcium
CAH	Congenital adrenal hyperplasia
CAII	Carbonic anhydrase II
CCD	Cortical collecting duct
CDC	Center for Disease Control
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
CV	Coefficient of variation
DCT	Distal convoluted tubule
DFTT	Dynamic functions test
ENaC	Epithelial sodium channel
EQA	External quality assurance
$Fe(HCO_3^-)$	Fractional excretion of bicarbonate
FTT	Failure to thrive
ILC	Interlaboratory comparison

ISE	ion selective electrode	QC	Quality control
kAE	Kidney anion exchanger	ROMK	Renal outer medullary potassium channel
kNBC	Kidney sodium-bicarbonate co-transporter	RT	Room temperature
LoB	Limit of the blank	RTA	Renal tubular acidosis
LoD	Limit of detection	SAG	Serum anion gap
M	Median	SBE	Standard base excess
N	Normal	SD	Standard deviation
NAE	Net acid excretion - titratable acid $+NH_4^+ - HCO_3^-$	SD _s	Standard deviation of a sample obtained by measuring the individual specimens within the sample
NCCLS	National Committee for Clinical Laboratory Standards		
NHE-3	Sodium-hydrogen exchanger	SD _{pool}	Standard deviation of sample consisting of pooled specimens
pCO ₂	Partial pressure of carbon dioxide		
pH	Negative logarithm, to the base ten, of the hydrogen ion concentration	SDI	Standard deviation index
		SID	Strong ion difference
pK	Dissociation constant	SIG	Strong ion gap
PCT	Proximal convoluted tubule	tCO ₂	Total carbon dioxide
PHA	Pseudohypoaldosteronism	TTKG	Transtubular potassium gradient = $\frac{[K^+]_s/[K^+]_u}{Osm_s/Osm_u}$
PHA2	Pseudohypoaldosteronism Type 2	UAG	Urine anion gap
PT	Proficiency testing	UOG	Urine osmolar gap
PTH	Parathyroid hormone	WNK1	With no lysine

A. Research Protocol for MMed Dissertation

Three documents (summary of study, a proposal and a detailed proposal) were submitted to the Research Ethics Committee and the Clinical Laboratory Sciences Departmental Research Committee for review as per their guidelines.

A.1 Summary of proposal for the design of external quality control program for uri- nary bicarbonate

A.1.1 Raison d'être

The diagnosis and management of renal tubular acidosis (RTA) is inexpensive. RTA may present with failure to thrive (FTT) and current opinion is that every child with FTT should be investigated for RTA.

One can calculate that in South Africa 500 000 newly diagnosed children with FTT should be identified per annum, of whom 250 000 are stunted or wasted. Thus, a need to screen for RTA exists. The benefit to the individual is both physical and psychological, the diagnosis may spare society the cost of more specialised investigation and, academically, it should facilitate the

determination of the epidemiology of this pathology.

Laboratories in South Africa should offer, but are reluctant to perform, the diagnostic tests (which include urinary bicarbonate) because the available methods/calculations – total CO_2 (tCO_2) and bicarbonate on a blood gas analyser – have not been validated for urine and no external quality assurance exists. By performing the test, the laboratory risks withdrawal of accreditation and cannot allow the clinician to make confident diagnoses.

The construction of an external quality assurance program for urine bicarbonate will allow laboratories to assist in the diagnosis of renal tubular acidosis. An external quality assurance program will facilitate screening of the at-risk population, will allow the epidemiology to be explored and the benefit of diagnosis to be determined. Furthermore, the test should be readily accessible through-out the country wherever blood gas analysers are used.

A.1.2 Study design

The study can be divided into two components. The first is experimental and the second is design.

The experimental component can further be divided into two. The first is the validation of the method and the second determines whether commercially available analytical grade ammonium bicarbonate and sodium bicarbonate can be used as external quality control material.

The design component uses the experimental data to design an external quality assurance program that is accessible, safe and cost effective for the country. Factors that will be considered are storage, transport, labour and

consequently cost.

A.2 Proposal for the design of external quality control program for urinary bicarbonate

Renal tubular acidosis is generally accepted to be a rare condition due to failure of the kidney to acidify the urine appropriately. Epidemiological data for the condition are, however, not available. The pathology should be considered in all cases of FTT[1].

Harrison[2] reports Nannan *et al.*'s[3] findings that in 1999, of the South African children in the age group 12 to 71 months, 48.7% were at least underweight. Of these children, 27.6% were either stunted or wasted. The census data for 2009 indicate that 10% of the South African population (5 000 000)[4] is under the age of 5 years. One should therefore anticipate that 250 000 new stunted or wasted children should be identified annually. Consequently, 250 000 children should be tested for RTA.

Of the tests used to diagnose and classify RTA[1], urine bicarbonate analysis cannot be implemented by the laboratory partly because the bicarbonate assay is not validated for urine and partly because no external quality assurance (EQA) program exists for urinary bicarbonate. The websites of all the EQA programs on a Center for Disease Control (CDC) list of EQA programs worldwide were used to determine whether an EQA schedule for urinary bicarbonate exists[5]. Such a schedule was not found.

Given the apparent need for the screening for RTA, based on the epidemi-

ology of FTT, it is proposed that an EQA program for urine bicarbonate be instituted. Given that (by definition) proficiency testing compares laboratory results to that of the group and/or with an assigned value[6], it is proposed that the same recovery experiment (using commercially available ammonium bicarbonate) be performed by different laboratories and the results used for interlaboratory comparison and/or comparison to the assigned value. Thus the recovery results would form the basis of performance assessment in the proficiency testing program.

The dissertation can be divided into two components. The first is validation which requires the characterisation of the analytical performance of the test methods. The second component is the design of an EQA program. Several questions have to be answered with regard to the EQA program.

For the validation of the method, standard protocols as documented by the Clinical and Laboratory Standards Institute (CLSI), formerly National Committee for Clinical Laboratory Standards (NCCLS), will be implemented. The protocols for analytical performance protocols are determination of the limit of detection (LoD)[7], the functional sensitivity[7], linearity[8] and intra-assay coefficient of variation (CV) at the clinical decision limits[9]. A recovery experiment[10, 11] and an interference experiment using sodium[11, 12] will determine the accuracy and specificity of the method. Finally a reference range[13, 14] for urinary bicarbonate will be established to determine population variation. Given that only 20 specimens will be used, it may not be possible to establish a reference range. Although one would anticipate that the variability will be minimal in which case the confidence intervals may be sufficiently small, allowing the use of only 20 specimens, it has already been demonstrated that the inter-individual variability is sufficiently large such that more than 20 specimens will be required[15].

An engineering design approach[16] will be used to construct an EQA program for urinary bicarbonate. The purpose of EQA is to ensure conformity of measurement[17]. Recovery experiments[11] using commercially available preparations of bicarbonate will form the basis of the EQA – thus ensuring conformity with a particular commercial manufacturer.

Before considering the bicarbonate compound to spike in the recovery experiment, it should be noted that at present two theories for acid-base phenomena are used in clinical chemistry[18–20]. The conventional theory is based on the Henderson-Hasselbalch equation[21] and the newer theory is the physico-chemical theory of acid-base[22]. The two acid-base theories predict different pH s depending on the compound added. This is important when calculating total CO_2 on an arterial blood gas analyser where total CO_2 is calculated from pH and the partial pressure of CO_2 .

It is proposed that a commercial preparation of ammonium bicarbonate (NH_4HCO_3) of known purity be used as the EQA substrate. There are, however, advantages to using sodium bicarbonate ($NaHCO_3$). Ammonium bicarbonate is volatile and requires storage in a cool, dry and well-ventilated place away from moisture, heat and sunlight. Ammonium bicarbonate may also be an irritant to the skin. These restrictions do not exist for sodium bicarbonate. The cost of both preparations is not significant but it is noted that sodium bicarbonate is less expensive. The sodium in $NaHCO_3$ does, however, have a significant effect in the physico-chemical theory and it also affects the value of the dissociation constant (pK) of H_2CO_3 on which the Henderson-Hasselbalch equation depends[23, 24]. Thus, one of the questions to be answered is whether using $NaHCO_3$ as EQA material as compared to NH_4HCO_3 is different. The data generated will be used to design and cost

an EQA program for urine bicarbonate which will be available for the country.

There are at least two methods used to determine the bicarbonate. The first uses a blood gas analyser and the Henderson-Hasselbalch equation to determine the bicarbonate (HCO_3^-) based on the pH , pCO_2 and pK [25]. The second method acidifies the urine converting all the HCO_3^- to CO_2 and then measuring the total CO_2 (tCO_2) [26]. These methods must be compared to determine whether the EQA results are comparable. The latter method is used by the Beckman-Coulter DxC[®] analyser evaluated in this document. The latter method does not depend on pH and should thus be independent of acid-base theory.

A.3 Details of test validation and external quality assurance

The experiments to be performed are divided into

1. determining the analytical performance and
2. design of external quality assurance.

The analyser to be used for the experiments is the Beckman-Coulter DxC[®].

A.3.1 Determining the analytical performance

Thirty millilitres of mid-stream urine from each of 20 healthy adult control subjects will be collected. The sample standard deviation (SD_S) [9] of bicarbonate will be determined based on these 20 specimens within the sample. The 95th centile of these results will be used to determine the limit of the blank (LoB) [7]. Five millilitre aliquots of the 20 specimens will then be pooled and the standard deviation of the pooled sample (SD_{pool}) will be determined by measuring the pooled sample 60 times [7]. The limit of detection

(LoD) will be calculated from SD_{pool} and LoB[7].

The baseline bicarbonate levels of the specimens were measured to determine the LoB. The 20 specimens will be spiked with a bicarbonate compound such that the bicarbonate is at double the clinical decision limit, the clinical decision limit (27 mmol/L as calculated in Appendix D4.1) and half the clinical decision limit – where the clinical decision limit is defined by a fractional excretion of bicarbonate of 5%[1]. The resultant bicarbonate in the specimens will be measured immediately and aliquots of these specimens will be stored at 4°C and room temperature (RT). Each of these stored specimens will be measured at 2 h and at 1 day. The bicarbonate in the individual specimens will be measured and the paired student t -test will be used to determine whether a significant change in the (temperature and storage-time stratified) samples has occurred. A less stringent reference change value analysis will also be performed. The standard deviation at the clinical decision limits will thus have been established and the stability at the above bicarbonate concentrations. A power function will be fitted to the data for the tests performed immediately (unspiked, spiked with half the decision limit, spiked with the decision limit and spiked with double the decision limit) in order to estimate the concentration at which the CV is 20% – the functional sensitivity[7]. Only 15 specimens will be tested at double the decision limit because the most important function of this level is to determine the linearity. Furthermore, the spiked specimens in which the bicarbonate is analysed immediately will only be aliquoted into different temperature groups after the initial measurement. The number of specimens analysed at time $t = 0$ stratified by concentration is summarised in Table A.1 below.

It is recognised that the 20 specimens analysed at 0 bicarbonate are included in the calculation of the LoB described above. Thus, only an additional 50

Fraction of bicarbonate decision limit	0	0.5	1.0	2.0
Number of specimens in each sample	20	20	20	15

Table A.1: NUMBER OF SPECIMENS TESTED, INITIALLY, FOR BICARBONATE CONCENTRATION – STRATIFIED BY FACTOR OF DECISION LIMIT

specimens will be analysed initially at these bicarbonate concentrations.

Given that the stability at zero bicarbonate concentration will not be determined, only the half critical limit sample, the critical limit sample and double the critical limit sample will be analysed at 2 *h* and 1 day. The tests to be analysed are tabulated in Table A.2 below. Thus a total of 160 specimens will be processed.

	4°C	RT
2 h	20	20
1 day	20	20

Table A.2: NUMBER OF SPECIMENS FOR THE DECISION LIMIT AND HALF DECISION LIMIT – STRATIFIED BY TEMPERATURE AND TIME OF ANALYSIS
RT: room temperature

The final component of the stability experiment is the number of specimens for the concentration that is double the clinical decision limit. Fifteen specimens will be analysed at $t = 0$. Four aliquots of each specimen will be analysed at 4°C and RT at 2 *h* and 1 day for a total of 75 specimens – 15 of which have already been tallied in Table A.1.

The data used to determine the stability above will also be used to establish linearity to double the clinical decision limit[8]. Thus only the interference experiment remains to complete the establishment of the analytical performance.

A.3.2 Design of the external quality assurance program

The first experiment will determine whether conformity exists between the commercially manufactured (and established) amount of ammonium bicarbonate and the amount determined by our analyser. The data for the tests performed immediately (unspiked, spiked with half the decision limit, spiked with the decision limit and spiked with double the decision limit) will be used in a recovery experiment[11]. The data will be analysed using linear regression, absolute and relative difference plots and paired student *t*-tests.

The same recovery experiments will be performed using sodium bicarbonate spiked into the specimens at the same concentration as the equivalent ammonium bicarbonate. Thus 20 specimens at each of three levels (unspiked, spiked with half the clinical decision limit and spiked with the clinical decision limit) and 10 specimens spiked with double the clinical decision limit will have recovery experiments performed on them[11]. The results will be compared directly to the manufacturer using linear regression, absolute and relative difference plots and paired student *t*-tests as above. The paired student *t*-test will also be used between the ammonium bicarbonate recovery results and the sodium bicarbonate recovery results to determine whether a difference exists between these samples.

A.3.3 The interference experiment

Sodium will be tested in the interference experiment[12]. Sodium has been selected because it alters the pK in the Henderson-Hasselbalch equation[23] and is a strong ion in the physico-chemical theory of acid-base equilibria[22]. The data from the EQA comparison described above will be used for the sodium interference experiment. The difference will be compared to the ac-

ceptable difference as described by Westgard[11]. The more stringent criteria of the NCCLS will also be used[12].

A.3.4 Costing for the experiment

All purchases will be from Sigma-Aldrich[®]. The cost of 1 *kg* analytical grade ammonium bicarbonate (product number 11213-1KG-R) is *R* 176,00[27]. The cost of 1 *kg* analytical grade sodium bicarbonate (product number 13433-1KG-R) is *R* 160,00[27]. An arbitrary figure of *R* 100.00 has been assigned for miscellaneous consumables such as wooden tongue depressors.

The total number of tests performed will be 410. The allocation of tests is as indicated below. Twenty tests will be performed to determine the LoB and 60 more tests to establish the LoD and CV. Thus, for the basic analytical performance, 80 tests will be performed.

The stability analysis will require a total of 295 tests. Given that 20 of the tests are used for the LoB, only an additional 275 tests will be performed for the stability analysis.

One hundred and thirty tests will be performed for the EQA analysis but more than half of these tests will form part of the stability experiment. Thus, only an additional 55 tests will be required to complete the EQA analysis.

A total of 130 tests will be required to perform the interference experiments but these will all be part of the stability experiment and thus no additional tests will have to be performed.

The cost of an individual test is *R*35,39. Thus the cost of performing

the experiments is $R14\,509.90$. The cost of the consumables is $R436$. The anticipated total cost is thus $R14\,945.90$.

A.3.5 Operating procedure

A table will be constructed with specimen numbers as first column, concentration on the first row, temperature in the second column and time in the third column. An ice pack will be available for temporary specimen storage. Each patient sample will have five 1.5 ml microcentrifuge tubes labeled with a temperature (4°C or RT), a time (0 min , 2 h or 1 day), a concentration (half decision limit, decision limit or double decision limit) and a specimen number (Figure A.1).

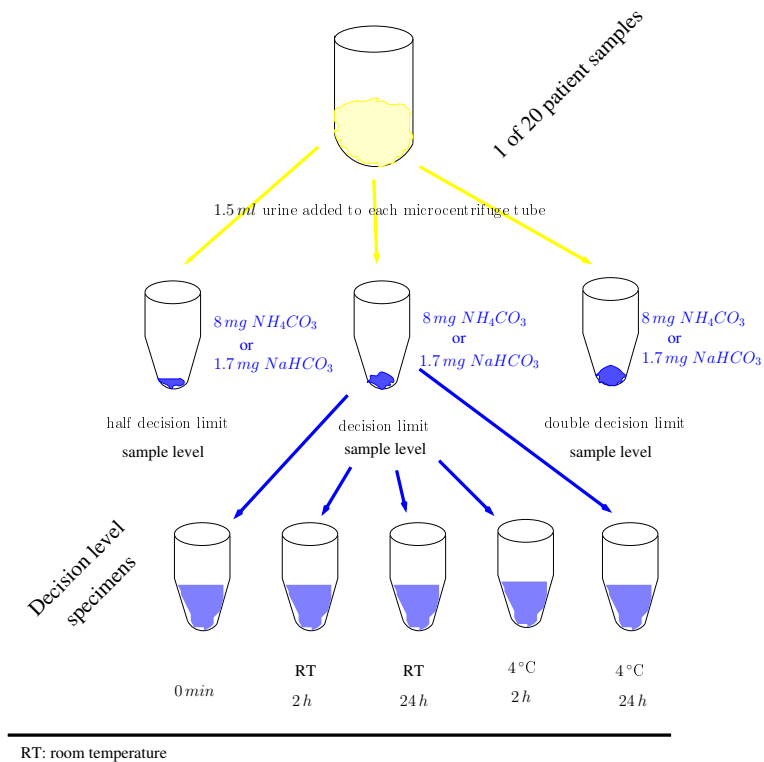


Figure A.1: URINE ALIQUOTS FOR $[\text{HCO}_3^-]$, STRATIFIED BY TEMPERATURE AND TIME – RT: room temperature

Note that the 0 min specimen does not have an assigned temperature. The 4°C microcentrifuge tubes will be stored on the ice pack.

It has been shown that it is not necessary to collect the urine under mineral oil if it is to be analysed within 2 h[15]. The wide variability of normal urine bicarbonate levels has been demonstrated[15]. It is therefore improbable that the 20 specimens will be adequate for a reference range. Nevertheless, for the reference range, a funnel will be used to collect urine directly into a test tube (minimising the exposed surface area) as previously described[15]. However, for the analytical and recovery experiments, mid-stream urines will be collected into standard urine collection containers and time will be allowed for equilibrium to be reached. During and after spiking (with ammonium bicarbonate or sodium bicarbonate), all specimens will be stored in volumetric flasks to minimise the exposed surface area.

From every subject (20) 30 ml of urine will be collected. Aliquots of 200 μ L will be used to measure tCO_2 immediately on the Beckman-Coulter DxC[®] analyser. One ml of urine from each sample will be pooled immediately. The bicarbonate concentration of forty 100 μ L aliquots of the pooled sample will then be measured.

For the half decision limit, decision limit and double decision limit sample levels, each of 3 aliquots of 7.5 ml of urine from each patient sample was spiked with either 8 mg, 16 mg or 32 mg of ammonium bicarbonate, respectively (Table A.3). This corresponds to 56 mg of ammonium bicarbonate per patient sample or 1.12 g of ammonium bicarbonate in total. From the above aliquots, 1.5 ml aliquots will be stored in the appropriate microcentrifuge tube. These specimens will be assayed at the appropriate times.

For the interference experiment, 1.7 mg of sodium bicarbonate will be dissolved in 1.5 ml urine for half the decision limit sample level, 3.4 mg sodium

Specimen Decision limit level	0.5	1.0	2.0	Mass/patient sample	Total
NH_4CO_3 added to urine (<i>mg</i>)	8	16	32	56	1 120
$NaHCO_3$ added to urine (<i>mg</i>)	1.7	3.4	6.8	11.9	238

Table A.3: MASSES OF NH_4CO_3 AND $NaHCO_3$ SPIKED INTO URINE TO ACHIEVE SPECIMEN DECISION LIMIT LEVELS

bicarbonate will be dissolved in 1.5 *ml* urine for the decision limit sample level and 6.8 *mg* will be dissolved in 1.5 *ml* for the double the decision limit sample level. Thus, for 20 specimens, a total of 238 *mg* of sodium bicarbonate will be used (Table A.3). Each of these specimens will be analysed once.

A.3.6 Test schedule

The tests will be performed on 3 weekends. On day one of the first weekend, 8 urine samples of 30 *ml* each will be collected and 1.5 *ml* aliquots will be used to measure tCO_2 immediately on the Beckman-Coulter DxC[®]. Thus 8 tests will be run on that day. One *ml* will be stored and subsequently pooled with the other patient specimens. The remaining urine will be stored as 3 aliquots of 7.5 *ml* each and one aliquot of 1.5 *ml*. Ammonium bicarbonate and sodium bicarbonate will be added as described in the operating procedure above and an additional 48 tests will be run on the spiked samples (24 for sodium bicarbonate and 24 for ammonium bicarbonate). Furthermore, 48 tests will be performed at 2 *h* (corresponding to 24 RT sodium bicarbonate specimens and 24 at 4°C). Thus a total of 104 tests will be performed on day one. On day two, the 1 day old specimens will be run at the 2 temperatures and will correspond to 48 tests. Thus 152 tests will be performed on the first weekend. The same procedure will be repeated on the second weekend with the same number of samples except that (in accordance with Table A.1) the last patient specimen will not be analysed at the double decision limit sample level (Table A.4).

Day	Immediate		2 h		24 h		Total
	unspiked	spiked	RT	4°C	RT	4°C	
1 st Sat	8	24 NH_4HCO_3 24 $NaHCO_3$	24	24			104
1 st Sun					24	24	48
2 nd Sat	8	23 NH_4HCO_3 23 $NaHCO_3$	23	23			100
2 nd Sun					23	23	46
3 rd Sat	4	8 NH_4HCO_3 8 $NaHCO_3$	8	8			36
3 rd Sun					8	8	16
Other							60
Totals	20	110	55	55	55	55	410

Table A.4: TABLE OF TESTS CONDUCTED STRATIFIED BY DAY AND TIME OF DAY: – Sat: Saturday, Sun: Sunday, RT: room temperature, Other: Limit of detection experiment

On the third weekend, the same tests as above will be performed but on 4 patient samples and none of these will have the double decision limit sample level. Thus 52 tests (36 on day one and 16 on day two) will be performed. The combined 20 samples that have been pooled will be analysed 60 times on the third weekend. Thus in total 410 tests will have been performed.

When considering the combined results (treating all the weekends as one), on the first day, 20 urine samples of 30 *ml* each will be collected. Aliquots of 1.5 *ml* will be used to measure tCO_2 immediately on the Beckman-Coulter DxC[®] analyser. One *ml* of urine from each sample will be pooled immediately. Sixty 1.5 *ml* aliquots of the pooled sample will then be measured. Thus initially a total of 80 tests will be analysed.

On the first day the remaining urine will be stored as 3 aliquots of 7.5 *ml* each and 1 aliquot of 1.5 *ml*. Ammonium bicarbonate and sodium bicarbonate will be added as described in the operating procedure. An additional 110 tests will be run immediately after adding the appropriate bicarbonate.

A total of 110 aliquots will be stored in microcentrifuge tubes at 4°C and 110 aliquots will be stored at RT. At 2 h, the bicarbonate of the 110 aliquots will be analysed. Thus on day one, 300 tests will be processed – 190 tests will be performed immediately and 110 at 2 h. On day 2, the remaining 110 samples will be processed. Thirty samples can be processed in 1 h and samples will be analysed in the same order as at time 0.

In the event that the LoD and LoB cannot be determined because the instrument cannot read to 0 analyte concentration, the 60 samples that would have determined the standard deviation near 0 will not be tested. Instead, the double decision limit sample level will be calculated on all 20 patient samples such that Table A.1 is modified to an alternate protocol (Table A.5).

Fraction of bicarbonate decision limit	0	0.5	1.0	2.0
Number of specimens in each sample	20	20	20	20

Table A.5: ALTERNATIVE PROTOCOL OF NUMBER OF SPECIMENS TESTED, INITIALLY, FOR BICARBONATE CONCENTRATION – STRATIFIED BY FACTOR OF DECISION LIMIT

Consequently, the tests performed will be changed such the original protocol (Table A.4) is modified to alternative protocol (Table A.6).

Day	Immediate		2 h		24 h		Total
	unspiked	spiked	RT	4°C	RT	4°C	
1 st Sat	8	24 NH_4HCO_3 24 NaHCO_3	24	24			104
1 st Sun					24	24	48
2 nd Sat	8	24 NH_4HCO_3 24 NaHCO_3	24	24			104
2 nd Sun					24	24	48
3 rd Sat	4	12 NH_4HCO_3 12 NaHCO_3	12	12			52
3 rd Sun					12	12	24
Totals	20	120	60	60	60	60	380

Table A.6: ALTERNATE TABLE OF TESTS CONDUCTED STRATIFIED BY DAY AND TIME OF DAY: – Sat: Saturday, Sun: Sunday RT: room temperature

A.3.7 Project summary

Twenty 30 *ml* mid-stream urine patient samples from adults are to be used. A total of 410 total carbon dioxide tests are to be performed to establish the analytical performance of the method where the analytical performance is defined by the CV, LoB, LoD, the functional sensitivity and interference. Stability is the sample property to be determined and the reference range is the population property to be determined.

The data collected will also be used to determine the analytical feasibility of using analytical grade (commercially available) ammonium bicarbonate and sodium bicarbonate as EQA material. The net result will allow the test to be performed by the laboratory to diagnose RTA. This will complete the experimental component of the dissertation.

The remainder of the dissertation will be devoted to the design (logistics) and costing of an EQA program for tertiary hospitals of the Western Cape.

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B. Literature Review

B.1 Objectives

The objective of the literature review can broadly be divided into three categories: defining renal tubular acidosis (RTA), identifying an at-risk population, and laboratory investigations to diagnose and classify RTA.

It will be shown that the determination of the fractional excretion of bicarbonate and therefore the measurement of urinary bicarbonate is essential for the classification of RTA.

The current clinical acid-base theories will briefly be introduced to explain why the bicarbonate compound used for the EQA program may affect the recovery of HCO_3^- . It is the potential discrepancy in bicarbonate recovery that is the motivation behind the comparison between Na_2HCO_3 and NH_4HCO_3 that will be evaluated as possible substrates for the EQA program.

B.2 Search strategy and quality criteria

The keywords "renal tubular acidosis" were paired with either "interlaboratory comparison", "proficiency test", "acid-base", "classify", "epidemiology", "failure to thrive", "stones" or "quality control" to identify 841 research or

review articles in the Pubmed database.

B.3 Renal tubular acidosis

B.3.1 Introduction

Renal tubular acidosis is defined, phenomenologically, as persistent hyperchloraemic metabolic (low bicarbonate) acidosis with inappropriately alkaline urine due to inability of the kidney to secrete acid (out of proportion to any loss of glomerular filtration rate, should such a loss exist)[1, 2].

In 1935 the first description, consistent with the above definition, reports 6 cases (from a cohort of 850 autopsies) of children with FTT and renal calculi all dying between the ages of 5 and 11 months [3, 4]. Importantly, the description did not include a normal anion gap metabolic acidosis with failure of urine acidification. Independently, in 1936, a syndrome of persistent hyperchloraemic acidosis (without excessive diarrhoea or vomiting), not responding to fluid therapy, associated with renal calculi and FTT was described[5]. RTA in adults was described[6] followed by an explanation of (and recognition of the association between) the triad of metabolic acidosis, inappropriately alkaline urine and nephrocalcinosis/nephrolithiasis in 1946[7–9]. The term RTA was introduced in 1951[8, 10].

The complexity and confusion of classification of RTA will be demonstrated under Section B.3.2. This dissertation proposes that the origin of the confusion is the attempt to establish a classification based on pathology as is conventional. This review will construct a classification based on clinical presentation and investigation which should be less confusing. Then pathologies will be associated with the subcategories of classification.

B.3.2 Historical taxonomy

The classification of RTA has changed with time. Initially RTA was classified as primary or secondary[11] – the former an isolated defect in renal tubular acidification and the latter associated with additional pathology. Furthermore, primary RTA was considered to be due only to failure to secrete H^+ appropriately[12, 13] whereas secondary RTA could be due to insufficient HCO_3^- reabsorption[14]. The publication of two case reports of primary RTA due to insufficient renal HCO_3^- reabsorption changed the classification to proximal RTA (with failure to reabsorb HCO_3^-) and distal RTA (with failure to establish adequate gradients of H^+)[14, 15]. Thus the classification was based on presumed histological/anatomical location of the defect[16, 17]. Concomitantly, RTA was classified as Type I, II and III based on the fractional excretion of HCO_3^- vs plasma HCO_3^- [18]. It should be recognised that the latter classification is independent of pathology/histology and consequently the two classification systems are not necessarily interchangeable[18]. Nevertheless, by 1972 convergence/reconciliation of the above classification systems had been achieved[19–21] and is summarised in Table B.1. Type III

Histological Classification	Bicarbonate Classification	Synonymous Terms
Distal RTA	Type I	Classic RTA, Gradient RTA
Proximal RTA	Type II	Bicarbonate-wasting RTA, Rate RTA
	Type III	Dislocation RTA

Table B.1: SYNONYMOUS TERMS AND ANALOGOUS CATEGORIES IN THE CLASSIFICATION OF RTA IN 1972 – RTA: renal tubular acidosis

RTA was considered a hybrid of Types I and II[21].

By 1982 the Type III classification (urine biochemistry with features of Type I and II) had been abandoned as being either transient or not a distinct entity from Types I or II – thereby unifying the classification systems[22, 23]. With the identification of carbonic anhydrase Type II (CAII) deficiency as a

distinct/primary RTA manifesting with biochemical features of Types I and II, the classification Type III RTA was re-introduced[24–26].

By 1997, Type IV RTA was broadly recognised[22, 27, 28] as a separate primary RTA due to hypoaldosteronism or pseudohypoaldosteronism (PHA). Thus, the four broad categories of RTA are summarised in Table B.2[26, 29]. The minimal investigations to distinguish between them are: urine pH , fractional excretion of bicarbonate($FeHCO_3^-$) after bicarbonate loading and serum potassium as indicated (Table B.2). Type II (in *italics* in Table B.2) is considered the only example of proximal RTA.

Investigation	Type I	<i>Type II</i>
Urine pH	≥ 5.5	< 5.5
$FeHCO_3^-$	$< 5\%$	$10 - 15\%$
Serum K^+	$\downarrow/N/\uparrow$	\downarrow/N
	Type III	Type IV
Urine pH	≥ 5.5	< 5.5
$FeHCO_3^-$	$5 - 15\%$	$5 - 10\%$
Serum K^+	\downarrow/N	\uparrow

Table B.2: CLASSIFICATION OF RTA IN 2002 AND BASIC URINE AND SERUM INVESTIGATIONS FOR DIAGNOSIS – derived from [26], RTA: renal tubular acidosis, $FeHCO_3^-$; fractional excretion of bicarbonate

Type III has been defined as all forms of RTA with biochemical features of both Type I and II[1, 30] or as only CAII deficiency[26, 31]. Viewing the classification from an alternate direction, CAII deficiency has been classified as Type I[17, 32, 33], Type II[8, 34], omitted from classification[30, 35], as Type III (a subgroup of Type I[26]) or Type III (distinct from Type I)[31, 36].

Furthermore, for completeness, each of the 4 categories of RTA can be subdivided aetiologically into primary and secondary. Type I can be complete (normal anion gap metabolic acidosis) or incomplete (no acidosis and normal serum bicarbonate), depending on whether dynamic function tests

(DFTT) are required to demonstrate RTA[32], and primary Type II can be isolated (no aminoaciduria) or associated with Fanconi syndrome[30] (a generalised aminoaciduria). If hereditary, RTA can be autosomal recessive or dominant[17, 30, 36, 37]. These subcategories and investigations are discussed in Sections B.3.5 and B.3.8.

A basic algorithm (compiled from reported results of investigations[26, 29]) for distinguishing between the general categories of RTA is provided in Figure B.1. Diarrhoea is omitted in the algorithm of Figure B.1 but is

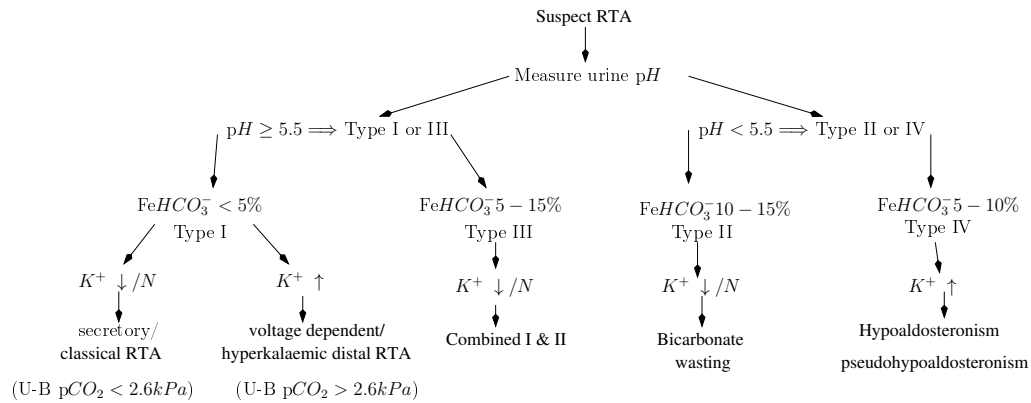


Figure B.1: BASIC ALGORITHM FOR DISTINGUISHING BETWEEN THE CATEGORIES OF RTA – compiled from [26, 29], RTA: renal tubular acidosis, FeHCO_3^- : fractional excretion of bicarbonate, pCO_2 : partial pressure of CO_2 , U-B pCO_2 : urine to blood pCO_2 gradient

included in the more comprehensive acidosis algorithm (Figure B.7).

B.3.3 Clinical presentation and basic investigation

Clinically, the more common RTA I and II can present as growth retardation, FTT, polyuria, polydipsia[33] or constipation[34]. Investigation may reveal a normal anion gap metabolic acidosis, refractory rickets/osteomalacia or hypokalaemia[26, 29]. The original RTA described is classical/distal/Type I RTA and the more severe autosomal recessive form is associated with sensorineural deafness and nephrocalcinosis/nephrolithiasis[17].

A Type III finding on investigation can either be secondary or primary. All the known secondary, and most of the primary forms, reduce to Type I or II[23]. The only known primary cause for Type III RTA (that does not reduce to Type I or II exclusively) is the rare CAII deficiency[26, 38]. Clinically, CAII deficiency is distinct – presenting with conductive deafness[17], osteopetrosis, cerebral calcification, mental retardation and biochemical features of both Type I and II (renal bicarbonate wasting and failure of H^+ secretion)[24, 25]. Biochemical Type III in the absence of the clinical manifestations of CAII deficiency will reduce to either Type I or II[23]. Given that CAII deficiency is biochemically intransient, here only CAII deficiency will be classified as Type III[26]. Furthermore, given that CAII deficiency is clinically distinct, Type III will be classified separately[31, 36], as originally intended[18].

Type I FTT, growth retardation refractory rickets/osteomalacia autosomal recessive: nephrocalcinosis, sensorineural deafness	<i>Type II</i> <i>FTT, growth retardation</i> <i>refractory rickets/osteomalacia</i> <i>secondary: Fanconi syndrome</i> <i>on investigation</i>
Type III mental retardation, conductive deafness, osteopetrosis, cerebral calcification	Type IV primary: asymptomatic PHA2: hypertension secondary: features of primary aetiology eg. ambiguous genitalia, salt-wasting

Table B.3: CLINICAL PRESENTATIONS IN RTA TYPES – FTT: failure to thrive, PHA2: pseudohypoaldosteronism type 2

Type IV RTA consists of a heterogenous group of pathologies affecting the renin-angiotensin-aldosterone axis[28] and the effector renal proteins – mineralocorticoid receptor[39] and the epithelial sodium channel (ENaC)[26, 28]. Type IV RTA is usually asymptomatic and incidently identified biochemically as hyperkalaemic normal anion gap metabolic acidosis[26]. Pseudohy-

poaldosteronism Type II (PHA2 or Gordan's syndrome) presents with arterial hypertension[40], hyperkalaemia but normal glomerular filtration rate by definition[41]. The clinical presentations are summarised in Table B.3.

B.3.4 Epidemiology

Data on the prevalence of RTA in children with FTT or rickets could not be found. It was reported that 22% (95% confidence interval (CI) 11 – 36%) of a cohort of 46 osteoporotic patients had incomplete RTA I when diagnosed by the NH_4Cl DFTT. For the same cohort, 35%(CI 21 – 50%) were identified as having incomplete RTA by furosemide DFTT[42]. It was also reported that 20% of a cohort of 300 patients with nephrolithiasis had either inherited or acquired Type I RTA[1].

B.3.5 Pathology and molecular basis of disease

The histological classification is more convenient than the bicarbonate classification when considering the pathology. Briefly, in acid-base homeostasis, the kidney is responsible for reabsorbing filtered bicarbonate and generating bicarbonate from CO_2 . The latter is accomplished by buffering secreted H^+ generated from water and CO_2 with ammonia and phosphate – allowing bicarbonate generation.

B.3.5.1 The proximal tubule and primary inherited RTA II

The cells of the proximal convoluted tubule (PCT) are responsible for reabsorption of bicarbonate[8, 26, 43]. The cell is depicted schematically in Figure B.2. There is an adenosine triphosphate(ATP) independent mechanism of HCO_3^- reabsorption which is actuated by a transtubular Na^+ gradient (depicted in green) and an ATP dependent HCO_3^- reabsorption mechanism (depicted in blue)[8, 26, 43]. The latter appears to be less signif-

icant. Regarding the ATP independent mechanism, a luminal membrane sodium hydrogen exchanger(NHE-3) and a basolateral membrane kidney sodium bicarbonate co-transporter (kNBC) facilitate the secretion of hydrogen and the reabsorption of HCO_3^- , respectively, down the sodium concentration gradient[1, 26, 33, 34]. The ATP dependent mechanism of reabsorption requires a luminal hydrogen pump and a basolateral Na^+ / K^+ -ATPase(adenosine triphosphatase)[8, 26, 33, 43]. The sodium bicarbonate co-transporter (kNBC) is also referred to as anion exchanger 1 (AE1). In Figure B.2 the proximal tubular cell regulation of ammonia (via facilitated NH_4^+ transport through a Na^+ / K^+ -antiporter) is depicted[8, 44] in violet. It has also been reported that ammonia (NH_3) diffuses directly across the cell membrane[34, 45] or that the Na^+ / K^+ -antiporter facilitates NH_4^+ transport[45]. It was suspected that a mutation in SLC9A3 coding for NHE-

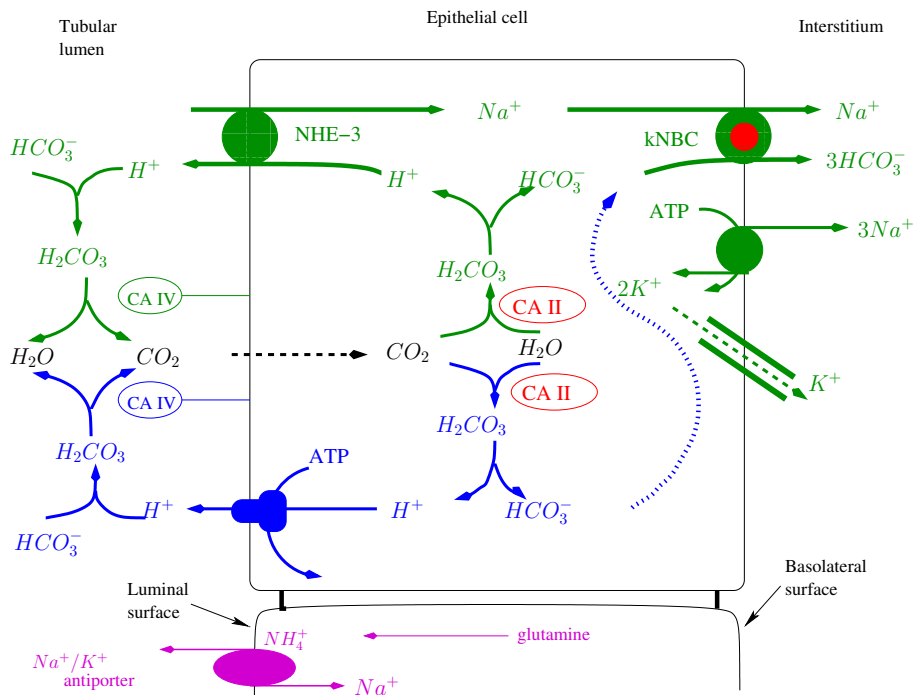


Figure B.2: PROXIMAL TUBULAR CELL H^+ REGULATORY PATHWAYS – [8, 44] NHE-3: sodium-hydrogen exchanger-3, CAII: carbonic anhydrase type II, kNBC: kidney sodium bicarbonate exchanger

3 is responsible for autosomal dominant RTA II[30] but as yet the mutation

has not been identified[36]. Autosomal recessive (AR) RTA II with ocular abnormalities is associated with mental retardation and has a mutation in SLC4A4 which codes for kNBC[30, 36]. The proximal tubular proteins with known inherited defects are depicted in red in Figure B.2.

B.3.5.2 The distal tubule, intercalated cells and primary inherited RTA I

The distal convoluted tubule (DCT) and cortical collecting duct (CCD) consist of at least two types of cell. The principal cells contain the amiloride sensitive ENaC and the intercalated cells are responsible for secreting either H^+ (α cells) or HCO_3^- (β cells)[8, 26]. The β -intercalated cells are of opposite polarity to the α cells (Figure B.3). Mutation in SLC4A1 which codes

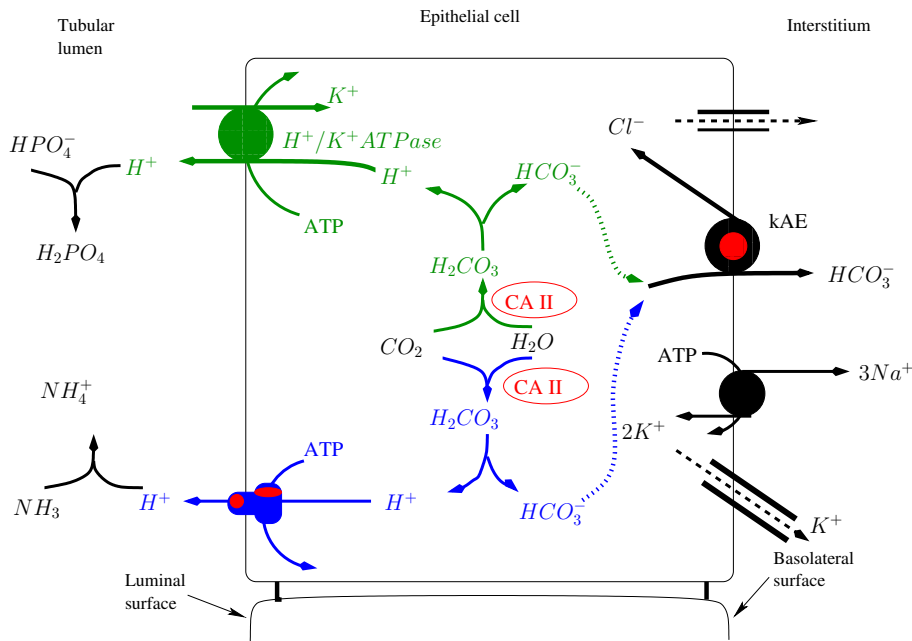


Figure B.3: REGULATION OF H^+ BY THE α -INTERCALCATED CELL COMPILED FROM – [8, 26] CAII: carbonic anhydrase II, kAE: kidney anion exchanger

for the kidney anion exchanger(kAE) is responsible for autosomal dominant (AD) RTA I. The known genetic causes for AR RTA I are all mutations in the H^+ ATPase. Mutation in ATP6V1B1 which codes for the β 1-subunit of the

H^+ ATPase[36, 37] produces RTA I with sensorineural deafness, and mutation in ATP6V0A4 which codes for the α 4-subunit of the H^+ ATPase[36, 37] produces RTA I without hearing loss.

B.3.5.3 The cortical collecting duct, the principal cell and primary inherited RTA IV

The principal cell contains ENaC and is regulated by aldosterone. The binding of aldosterone to the mineralocorticoid receptor induces both genomic and extra-genomic effects. In particular, in the α -intercalated cell of the DCT, the upregulation of the apical proton pumps is a non-genomic effect of aldosterone[45]. In contrast, the activation of ENaC is a genomic effect.

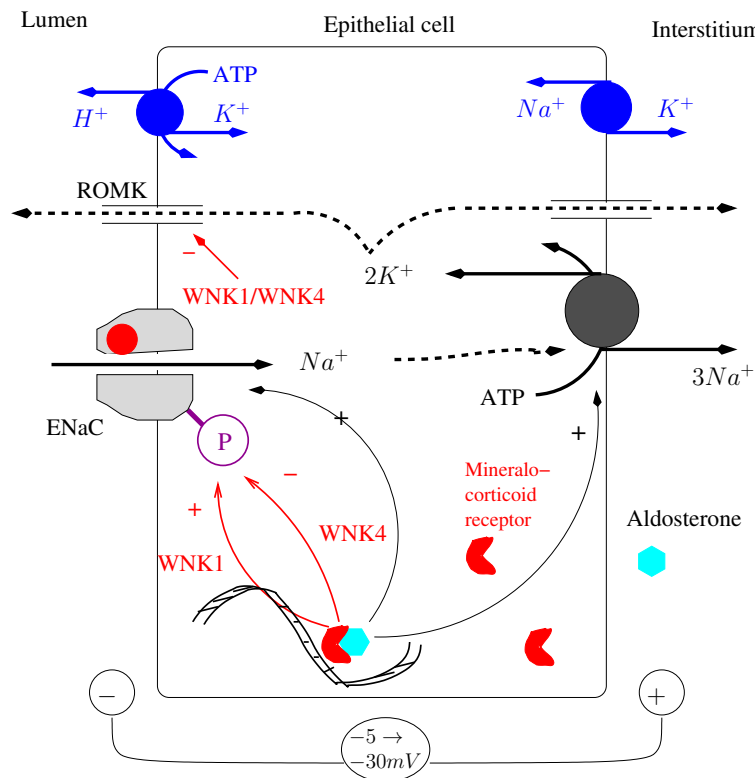


Figure B.4: ROLE OF THE PRINCIPAL CELL IN RTA IV – [46] ROMK: renal outer medullary potassium channel, WNK: with no lysine, ENaC: epithelial sodium channel

Figure B.4 is a schematic of the principal cell depicting the various PHA

defects – only the subcellular pathways relevant to the known causes of PHA are depicted[46]. Briefly, aldosterone diffuses across the cell membrane and binds to the intracellular mineralocorticoid receptor. One of the reasons for the mineralocorticoid specificity is the inactivation of cortisol to cortisone by β hydroxysteroid dehydrogenase 2[47]. The genomic effects include upregulation of ENaC and the basolateral Na^+/K^+ -ATPase. The subcellular signal WNK1 (with no lysine where K is the symbol for lysine) phosphorylates and thereby activates ENaC while WNK4 dephosphorylates ENaC. Both WNK1 and WNK4 inhibit the luminal membrane renal outer medullary potassium channel(ROMK) by interacting with the scaffolding[48]. The principal cell promotes H^+ excretion indirectly by increasing the negative potential difference of the tubular lumen[8, 26, 29]. A H^+/K^+ -ATPase may also increase secretion directly but appears to be more important in K^+ homeostasis[26].

The primary PHA defects are: AR PHA I with defects in the genes coding for the ENaC, AD PHA I with defects in the genes coding for the mineralocorticoid receptor, AD PHA II subtype *a* associated with a locus on chromosome 1q31-q42, AD PHA II subtype *b* due to mutations causing underexpression of WNK4, AD PHA II subtype *c* due to mutations causing overexpression in WNK1, and PHA for which no gene locus has been identified[46].

B.3.5.4 The loop of Henle and ammonium secretion

The loop of Henle contains the furosemide sensitive sodium-potassium-chloride symporter which is capable of reabsorbing NH_4^+ instead of potassium[8, 44]. The reabsorbed NH_4^+ is the source of the NH_3 required for buffering the distally secreted H^+ . It is, however, not obvious how NH_3 is delivered distally as opposed to NH_4^+ [28]. It is also suggested that the NH_3 diffuses directly across the membrane[28, 34, 45]. The two mechanisms (which may not be

mutually exclusive) for the delivery of ammonia are depicted in Figure B.5.

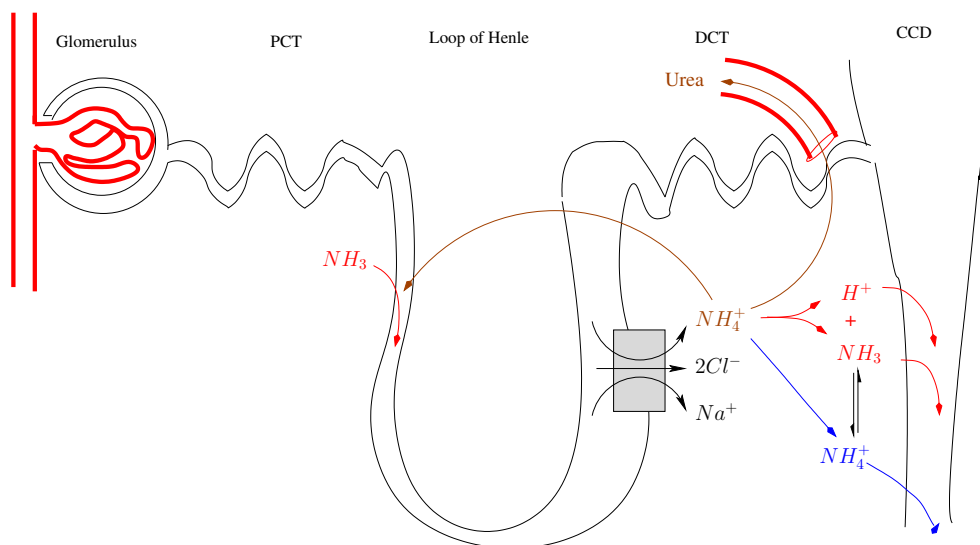


Figure B.5: THE FATE OF AMMONIA AND AMMONIUM IN THE NEPHRON – modified from [28] PCT: proximal convoluted tubule, DCT: distal convoluted tubule, CCD: cortical collecting duct

There are at least two theories regarding the role of NH_4^+ . The traditional view is that NH_3 consumes secreted H^+ in the distal nephron (forming NH_4^+) and thereby allows regeneration of HCO_3^- . The traditional view is depicted on the left in Figure B.6. The alternate view is that the formation of NH_4^+ consumes NH_3 that would otherwise undergo hepatic carbamylation which, in turn, consumes HCO_3^- [49]. Thus NH_4^+ conserves HCO_3^- – the right side of Figure B.6.

B.3.5.5 CA II deficiency and RTA III

CA II is present both proximally and distally within the nephron (Figures B.2 and B.3). CA II deficiency has been described in patients on most continents but the majority of the cases appear to be due to a founder effect in an Arab tribe that migrated to North Africa [38]. The classification of RTA, with some of the primary causes, is summarised in Table B.4.

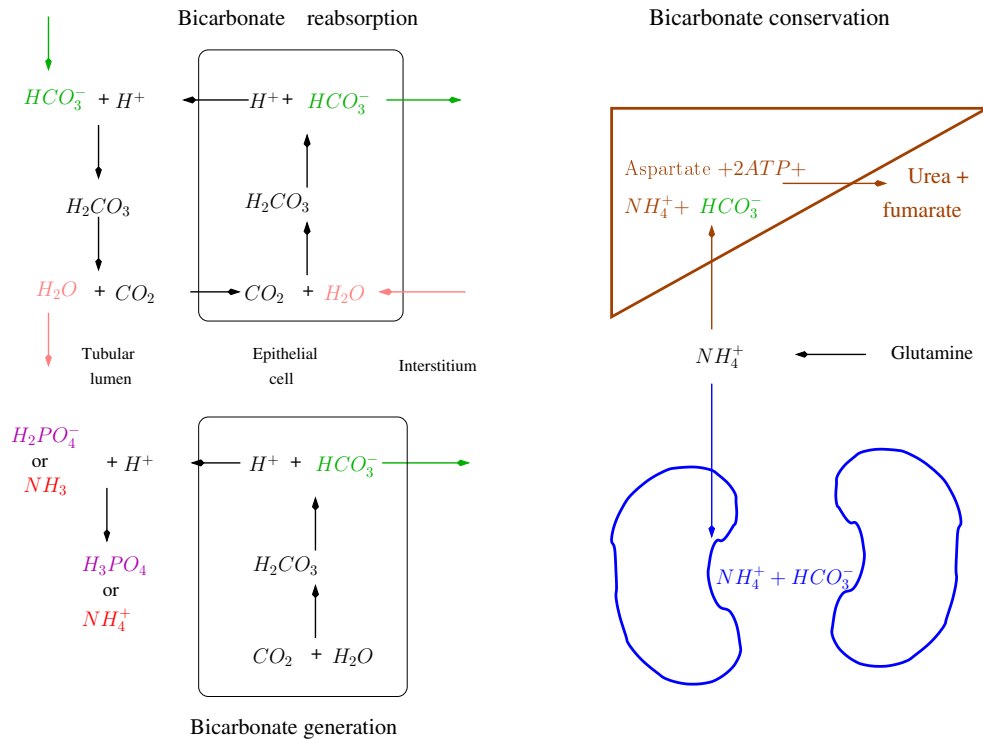


Figure B.6: POTENTIAL MECHANISMS FOR THE ROLE OF NH_3 IN ACID-BASE REGULATION

<p>Type I secretory AD RTA I, AR RTA I voltage dependent</p>	<p>Type II <i>insufficient HCO_3^- reabsorption</i> <i>AD RTA II, AR RTA II with deafness,</i> <i>AR RTA II without deafness</i></p>
<p>Type III carbonic anhydrase II deficiency</p>	<p>Type IV hypoaldosteronism PHA AD PHA I, AR PHA I, AD PHA II b, AD PHA II c, AD PHA II a, PHA III</p>

Table B.4: CLASSIFICATION OF RTA AND SOME PRIMARY CAUSES
 – RTA: renal tubular acidosis, AD: autosomal dominant, AR: autosomal recessive, PA: pseudohypoaldosteronism

B.3.6 Pathological basis of classification

The classification system in Table B.2 is based on biochemical investigation correlated with the distinct clinical findings in Table B.3. This section correlates taxonomy and pathophysiology and should be interpreted together with Section B.3.5.

Type I RTA is due to impaired H^+ secretion with a secondary defect in NH_4^+ excretion[8, 26]. The defect in renal collecting duct α -intercalated cell H^+ secretion can either be direct or indirect where a direct defect is present in the H^+ -ATPase on the apical membrane and an indirect defect is on the basolateral membrane HCO_3^-/Cl^- exchanger (AE1). Both the direct and indirect failure to secrete H^+ are defined as classical or secretory Type I RTA[26]. Type I RTA is not known to involve the H^+/K^+ -ATPase, also present on the α -intercalated cell, which may explain the possibility of hyperkalaemia (Table B.2).

Non-secretory defects manifesting as Type I RTA are either gradient-, low buffer- or voltage-dependent. Gradient-dependent RTA is defined by a failure to maintain an H^+ gradient (H^+ -ATPase is intact) due to ‘back-leak’ of H^+ . Gradient-dependent RTA is often secondary to amphotericin B treatment.

A second non-secretory Type I defect is ‘low buffer’ Type I RTA. The ‘low buffer’ type defect is defined by proximal tubular NH_4^+ secretion failure (seen in nephrocalcinosis and chronic interstitial nephritis)[26].

The H^+ -ATPase of secretory Type I RTA is also influenced by the electrochemical gradient across the cell membrane – which is maintained by the

principal cells. It is necessary for H^+ to be secreted along the distal lumen electronegative transepithelial gradient. A defect in this transepithelial electrogradient is a voltage-dependent defect and is due to impaired Na^+ transport – secondary to obstructive uropathy, salt-losing congenital adrenal hyperplasia(CAH), administration of drugs (lithium, amiloride, trimethoprim, pentamidine). Presumably the consequent impairment in K^+ transport accounts for the hyperkalaemia of voltage-dependent Type I RTA.

<p>Type I SECRETORY classical RTA apical H^+-ATPase or basolateral AE1</p> <p>NON-SECRETORY non-hyperkalaemic gradient dependent H^+ ‘back-leak’ low buffer type impaired proximal NH_4^+ secretion</p> <p>hyperkalaemic voltage dependent principal cell Na^+ transport defect with secondary K^+ transport derangement</p> <p>Type III CA II deficiency</p>	<p><i>Type II</i></p> <p><i>impaired proximal tubular bicarbonate reabsorption</i></p> <p>Type IV</p> <p>Hyperkalaemic Type 4 impaired ammoniogenesis due to hyperkalaemia impaired bicarbonate reabsorption (> I, < II)</p>
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Table B.5: TAXONOMY-PATHOPHYSIOLOGY CORRELATION IN RTA – compiled from [26]. AE1: anion exchanger 1, CA II: carbonic anhydrase II, ATPase: adenosine triphosphatase

RTA II is secondary to impaired HCO_3^- reabsorption while RTA III is a CA II deficiency

RTA IV is associated with hypoaldosteronism and PHA. Although the distal tubular cells have H^+/K^+ -ATPase, these are primarily responsible for

K^+ reabsorption rather than H^+ excretion – thus the impaired H^+ secretion is not the cause of the acidosis. Rather, hyperkalaemia inhibits ammonia reabsorption at the ascending limb of the loop of Henle[28]. The acidosis is due to impaired proximal tubular NH_4^+ secretion – which buffers the secreted H^+ . There is a consequent HCO_3^- reabsorption defect which is intermediate between RTA I and RTA II. Thus RTA IV is due to hyperkalaemia. Thus, other than the defects in the renin-angiotensin-aldosterone axis, any cause of hyperkalaemia will result in RTA IV[26] such as chronic renal failure, potassium-sparing diuretics, heparin, insulin antagonists, β -adrenergic antagonists, α -adrenergic agonists, digitalis, succinylcholine. Thus in RTA IV, hyperkalaemia is causal whereas in voltage dependent Type I RTA, hyperkalaemia is a consequence.

The taxonomy-pathophysiology correlation is summarised in Table B.5.

B.3.7 Diagnostic algorithms

The biochemical results of the basic algorithm for RTA diagnosis (Figure B.1) are summarised in Table B.2 and these features are common to all presentations. The pathology should enter the differential diagnosis of a metabolic acidosis and should be isolated (or recognised as distinct) based on the serum anion gap (SAG)[33, 50] and the urine anion gap (UAG)[8, 33, 34, 51] or urine osmolar gap (UOG)[8, 51] in the acidosis algorithm (Figure B.7). An IgG paraprotein can induce either a low normal or negative SAG and RTA[50]. In neonates, UAG should be replaced with a DFTT[34] because ammonium generation matures postnatally.

Urine pH and $Fe(HCO_3^-)$ distinguish hyperkalaemic RTA Type I from Type IV RTA(Figure B.1). It is evident that Type IV RTA may mask hy-

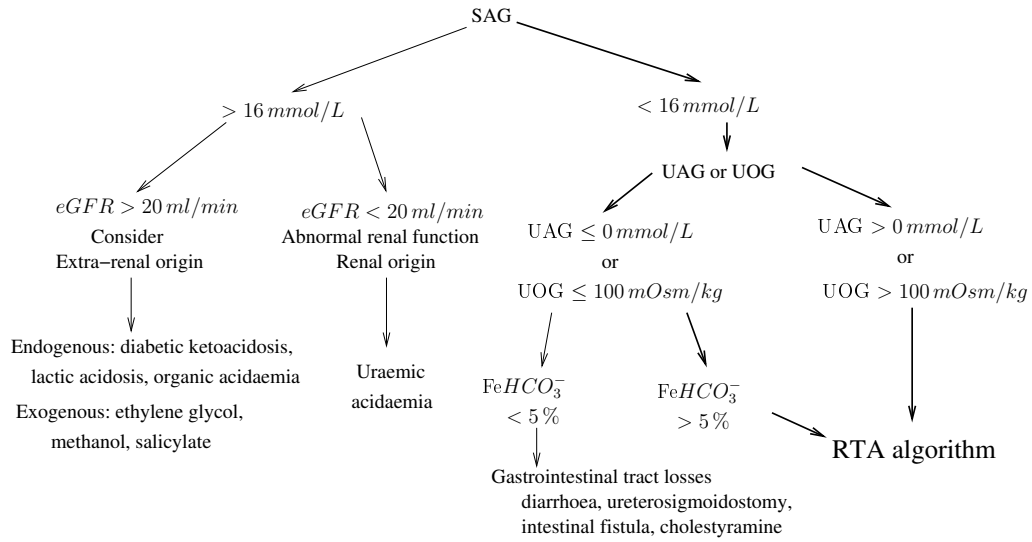


Figure B.7: DIAGNOSTIC ALGORITHM FOR CONFIRMING RTA IN METABOLIC ACIDOSIS – [33, 50] SAG: serum anion gap, eGFR: estimated glomerular filtration rate, UAG: urine anion gap, UOG: urine osmolar gap, $FeHCO_3^-$: fractional excretion of bicarbonate, RTA: renal tubular acidosis

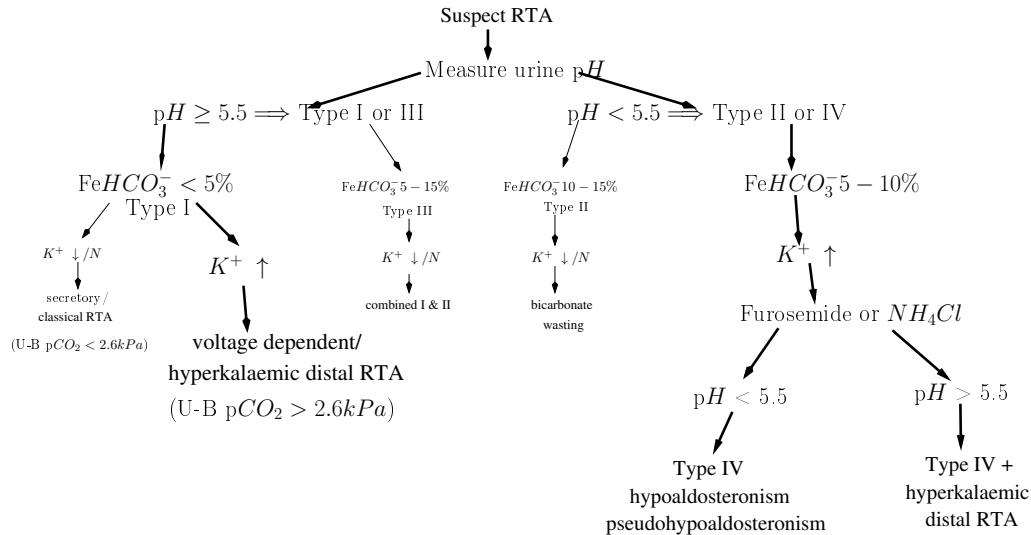


Figure B.8: HYPERKALAEMIC RTA DIAGNOSTIC ALGORITHM – ONLY INCREASED K^+ EMPHASIZED – [26, 29] RTA: renal tubular acidosis, $FeHCO_3^-$: fractional excretion of bicarbonate, pCO_2 : partial pressure of CO_2 , U-B pCO_2 : urine to blood pCO_2 gradient

perkalaemic RTA Type I[52]. Furosemide or NH_4Cl should stimulate the renin-angiotensin-aldosterone system in Type IV RTA – manifesting as a reduction in urine pH and urine $[K^+]$ ($[K^+]_u$). Conversely, in the presence of RTA and the furosemide/ NH_4Cl test, failure to reduce pH and $[K^+]_u$ implies hyperkalaemic Type I RTA[26]. The refined algorithm for hyperkalaemic RTA is depicted in Figure B.8[8].

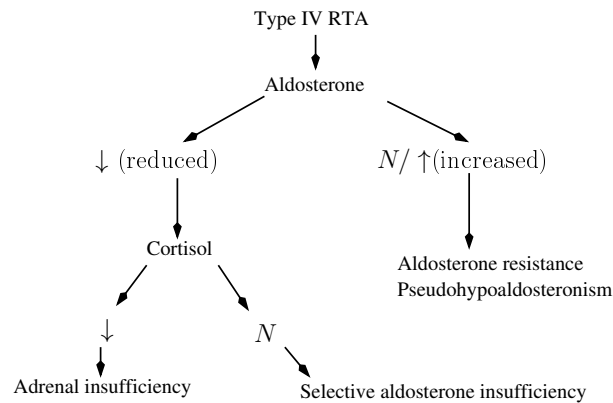


Figure B.9: RTA IV DIAGNOSTIC ALGORITHM[8]

RTA IV is due to hyperkalaemia. Primary causes of the hyperkalaemia include hypoaldosteronism and PHA. The direct method for distinguishing between them is aldosterone measurement[8]. A proxy measure of aldosterone activity is transtubular potassium gradient (TTKG)[26, 28]. The direct measure of aldosterone is preferred[53]. The algorithm to follow once RTA IV has been diagnosed is depicted in Figure B.9.

The definitive investigations for diagnosis are presented in Section B.3.8. A prominent feature of classical/secretory Type I RTA is nephrolithiasis and an algorithm has been compiled from reported data[54](Figure B.10).

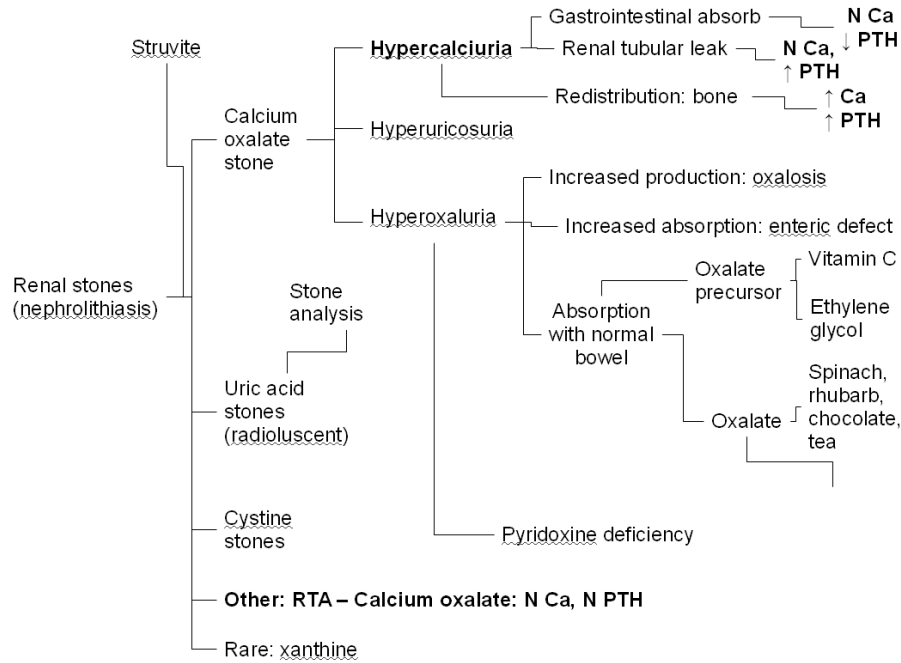


Figure B.10: DIAGNOSTIC ALGORITHM FOR RENAL STONES – compiled from [54] PTH: parathyroid hormone, Ca: calcium

B.3.8 Laboratory investigation

The laboratory diagnosis can be divided into three categories: diagnosing complete RTA and categorising the RTA, distinguishing between the subcategories of RTA, and DFTT to diagnose incomplete RTA. The molecular basis of the genetic RTAs is discussed in Section B.3.5 but the molecular diagnosis is not addressed in this review.

Arterial blood gas and serum Na^+ , K^+ , Cl^- , HCO_3^- analysis are used to determine whether a normal anion gap metabolic acidosis exists (Figure B.7). Once a hyperchloraemic metabolic acidosis is confirmed, urine and serum bicarbonate and creatinine, and urine pH , are determined to exclude diarrhoea as the cause of hyperchloraemic metabolic acidosis and to confirm the diagnosis of RTA (Figure B.7).

Sodium bicarbonate is administered to correct the serum bicarbonate be-

fore determining the $Fe(HCO_3^-)$ and thereby classifying the RTA (Figure B.1).

There are various DFTTs to diagnose incomplete RTA[8]. However, since there is no clinical management for this condition, it is debatable whether they are of clinical utility. The bicarbonate loading test is the definitive test for RTA II[8]. DFTT (furosemide or NH_4Cl stimulation tests) are, however, useful at detecting a hyperkalaemic RTA I being masked by RTA IV.

The furosemide stimulation test inhibits the reabsorption of K^+ by the $Na^+/K^+/2Cl^-$ co-transport system – increasing the distal Na^+ delivery[8, 26] and luminal electronegativity[26, 33]. The normal response is for the urine pH to decrease and K^+ excretion to increase – neither of these events occurs in voltage-dependent RTA I[8, 26, 33]. A urine $pH > 5.5$ after furosemide stimulation indicates hyperkalaemic RTA I. The furosemide can be delivered orally (40mg)[8] or intravenously (1mg/kg body weight)[26]. The urine pH should be measured half-hourly for up to 5 h, for the orally administered test, (or up to 3 h for the intravenous test) to determine the lowest pH . A $pH < 5.5$ is a normal response. The pH does not reduce to 5.5 in hyperkalaemic RTA. The NH_4Cl test is the definitive method for RTA I but NH_4Cl is unpalatable and requires an anti-emetic[8].

B.4 The physicochemical acid-base theory, RTA and bicarbonate proficiency testing

An introduction to clinical chemistry acid-base theories is provided in Appendix D1. Two theories dominate – bicarbonate-centric Henderson-Hasselbalch based approaches and the physicochemical approach by Stewart. Evidence

suggests that the only real difference between the two is that the latter considers the effect of non-bicarbonate buffers. Nevertheless, the effect appears to be sufficiently small so as not to make a difference to clinical decisions.

Bicarbonate based approaches measure bicarbonate indirectly from pH and pCO_2 in an *in vitro* environment. The approach has been criticised for not reflecting the *in vivo* environment[55, 56]. The interpretation of Stewart's approach suggests that bicarbonate is a dependent factor with regard to acid-base homeostasis and that consequently bicarbonate is a function of the difference between serum cations and anions – the strong ion difference(SID).

Traditional models of RTA emphasize the role of H^+ -pumps and transporters and HCO_3^- transporters but, as has recently been published, these pumps and transporters rely on cations (like Na^+) and anions (like Cl^-) to maintain electroneutrality and consequently do not refute Stewart's theory[31]. Thus, the compound selected for a potential proficiency testing program should not violate either theory. Sodium bicarbonate is a potential compound but affects SID in the physicochemical approach. Ammonium bicarbonate should in principle have the same effect in both theories.

B.5 Further research required

Additional genetic causes for RTA need to be determined. Although the mechanisms of the effect of transporters have been described, the regulation of these transporters remains to be determined. Without these regulatory mechanism data, neither the physicochemical acid-base theory nor the bicarbonate-centric theory can be disproved[31]. Elucidation of the regulatory mechanisms may refute at least one theory.

A proficiency testing program does not exist for urinary bicarbonate. Such a program would facilitate multicenter trials, allow comparison of laboratory results and allow epidemiological studies on the prevalence of RTA in various groups. The existence of proficiency testing programs would also allow the determination of the clinical sensitivity, specificity, and positive and negative predictive values of employing the diagnostic algorithms (Figure B.7).

B.6 Summary

RTA is a well described pathology but there is little epidemiological information on this condition. Many molecular causes have been elucidated. However, the regulation of the pathways has as yet not been clearly described. The study of the regulation of the molecular mechanisms of RTA may provide a comprehensive explanation of acid-base homeostasis (as opposed to a description).

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C. Manuscript

Title

DESIGN, IMPLEMENTATION AND EVALUATION OF INTERLABORATORY COM-
PARISON PROGRAM FOR URINE BICARBONATE

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Journal

ACCREDITATION AND QUALITY ASSURANCE

Keywords

Proficiency testing, interlaboratory comparison, urine bicarbonate, volatile substrate, accreditation, validation

C.1 Abstract

Urine bicarbonate analysis is required in the diagnostic workup of renal tubular acidosis (RTA) – which should be considered in wasted, stunted children. The expected incidence of stunting, in South Africa, is 250 000 per annum – establishing a need for urine bicarbonate testing. For accreditation, a laboratory requires external quality assurance for urine bicarbonate. The absence of proficiency testing for urine bicarbonate poses a problem.

Accreditation requires at least an interlaboratory comparison (ILC) program. Bicarbonate volatility hinders ILC. Here an ILC is designed based on the recovery of bicarbonate from freshly spiked urine samples.

The analytical performance of the Beckman-Coulter DxC[®] assay for total bicarbonate in urine is calculated and used to determine acceptable limits of an ILC. Sodium bicarbonate is selected as ILC substrate as a safer, cheaper and more soluble option than ammonium bicarbonate. This work is a case study in proficiency testing of unstable analytes.

The ILC uses difference in recovery of exogenous bicarbonate (to negate variation in endogenous bicarbonate) to compare laboratories. The results are analysed using standard deviation index plots and Youden plots.

The marginal administrative cost of the ILC is R4,64 per ILC cycle. The marginal costs of analyses are R4,64 on an arterial blood gas analyser and R17,64 on the Beckman-Coulter DxC[®].

C.2 Introduction

Renal tubular acidosis (RTA) is defined phenomenologically as persistent hyperchloraemic metabolic (low bicarbonate) acidosis with inappropriately alkaline urine due to inability of the kidney to secrete acid (out of proportion to drop in glomerular filtration rate – should such a drop exist)[1, 2]. The measurement of urine bicarbonate is required for the classification of RTA[3]

Clinical laboratories are accredited by authoritative peer-review bodies[4–6]. Several such authoritative bodies exist – accrediting by variable criteria[4–10]. Generally, medical laboratories endeavour to be accredited to ISO 15189 standards[5] and this allows the laboratory to select the tests to be evaluated and the scope of the evaluation. The National Health Laboratory Service in South Africa is accredited by the South African National Accreditation Service[6] and attempts to have all its tests accredited – the individual tests are listed on the accreditation certificate[11].

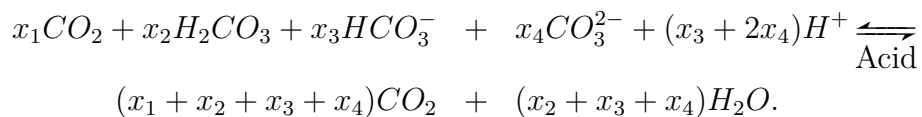
A requirement for test accreditation is that the laboratory should subscribe to a proficiency testing (PT) program[5, 6] for that test. No such program was identified for urine bicarbonate. ISO 15189 does allow for inter-laboratory comparison (ILC) - should PT be unavailable[5]. This dissertation designs an ILC program[5] (based on recovery experiments and a Clinical and Laboratory Standards Institute (CLSI) guideline[12]) for urine bicarbonate – allowing accreditation. Recovery is selected to avoid the problem of volatility.

The factors to be considered when selecting a bicarbonate compound are whether the compound violates relevant acid-base theories, stability, reproducibility of results, cost and ease of handling – solubility and safety. Two acid-base theories dominate in clinical medicine. The traditional Henderson-

Hasselbalch theory is the more widely used while the new physicochemical theory is favoured by anaesthetists and intensivists. The Henderson-Hasselbalch approach is bicarbonate-centric and, in principle, should not be affected by the bicarbonate compound selected[13]. The physicochemical model focuses on the strong ion difference (SID) in its interpretation of acid-base disturbances[14]. In the physicochemical approach only predefined cations are considered. Thus, depending on the cation in the compound spiked, the SID may be significantly changed. The compounds to be evaluated for the recovery experiments are ammonium bicarbonate (acceptable in both theories) and sodium bicarbonate (sodium, as a strong ion, should induce alkalosis in the physicochemical approach).

C.3 Materials and methods

Bicarbonate was measured as total CO_2 (the sum of all bicarbonate species) by a pCO_2 electrode on a Beckman-Coulter DxC[®] analyser. All bicarbonate species are converted to CO_2 by acidification (Figure C.1):



Total carbon dioxide (tCO_2) (on the DxC platform) has an analytical range of 5 – 50 $mmol/L$. The within-run imprecision is 1.5% at 28.99 $mmol/L$ and

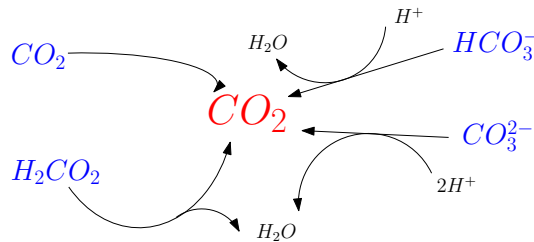


Figure C.1: THE EFFECT OF ACIDIFICATION ON BICARBONATE SPECIES

the total imprecision is 2.8% at 28.99 $mmol/L$. The resultant CO_2 is measured using a CO_2 ion selective electrode (ISE)[15].

The Bayer RapidLab[®] 348 blood gas analyser uses a pH ISE to calculate the hydrogen ion concentration directly and a pCO_2 ISE to calculate the actual HCO_3^- [16] by re-arranging the Henderson-Hasselbalch equation such that

$$HCO_{3act}^- = 0.031 \times pCO_2 \times 10^{(pH-6.1)}. \quad (C-1)$$

The total CO_2 can then be calculated as

$$tCO_2 = 0.031pCO_2 + HCO_{3act}^-. \quad (C-2)$$

Analytical grade ammonium bicarbonate (11213-1KG-R) and analytical grade sodium bicarbonate (13433-1KG-R) were purchased from Sigma-Aldrich[®]. Urine was not collected under mineral oil[17]. Twenty 1.5 ml aliquots of urine from 20 individuals (1 aliquot from each of the patient samples were tested for tCO_2 immediately and immediately after spiking with a bicarbonate compound. From each of the 20 individuals, 18 additional aliquots (from the original patient sample) of 1.5 ml were collected. Of the 18 aliquots, 3 sets (or samples) of 5 specimens were spiked with 1.6 mg (13.5 $mmol/L$), 3.2 mg (27 $mmol/L$) or 6.4 mg (54 $mmol/L$) of NH_4HCO_3 , respectively. Of the remaining 3 urine aliquots, each aliquot was spiked with 1.7 mg (13.5 $mmol/L$), 3.4 mg (27 $mmol/L$) or 6.8 mg (54 $mmol/L$) of $NaHCO_3$. The aliquots spiked with $NaHCO_3$ were analysed immediately – for comparison with NH_4HCO_3 aliquots that were analysed immediately. One of the NH_4HCO_3 quintuplicates was analysed for tCO_2 immediately, 2 were stored at room temperature and analysed at 2 h and at 24 h . The remaining 2 NH_4HCO_3 quintuplicates were stored on ice and refrigerated at 4°C before being anal-

ysed at 2 h and 24 h. One urine sample was spiked with NH_4HCO_3 (as described above) and aliquots (forming the quintuplicate) were acquired from the spiked sample (Figure C.2). All values of bicarbonate less than 5 mmol/L

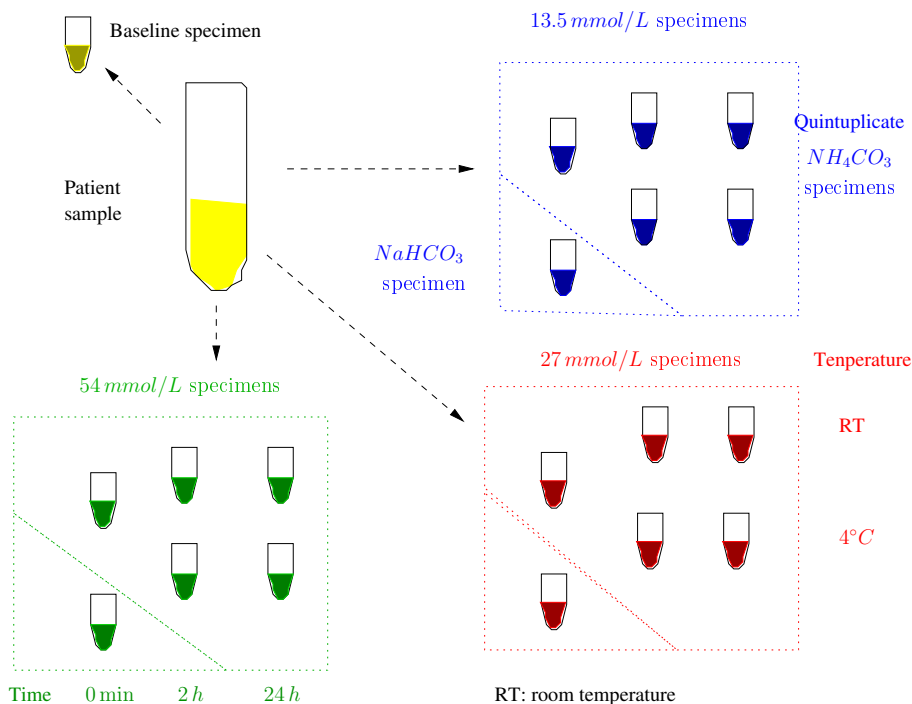


Figure C.2: THE ALIQUOTS OF THE ORIGINAL URINE PATIENT SAMPLE REQUIRED FOR SPIKING – stratified by compound, concentration, storage time and storage temperature, RT: room temperature

are reported as zero. Specimens with bicarbonate readings greater than 50 mmol/L were diluted 1:2 and re-analysed.

The results were used to determine the HCO_3^- stability in the sealed microcentrifuge tube, the precision of the instrument, the functional sensitivity of the assay, a reference range, and to compare the results for $NaHCO_3$, NH_4HCO_3 and the gravimetrically prepared HCO_3^- . The 24 h NH_4HCO_3 samples were also analysed on a Bayer Healthcare[®] Rapidlab 348 arterial blood gas (ABG) analyser.

Normal distributions were excluded using the Shapiro-Wilk test and QQ-

plots. Statistically significant differences were evaluated using either the paired student t -test or analysis of variance (ANOVA) if the sample analyte results were parametrically distributed or by the Wilcoxon test or Mann-Whitney U test if non-parametrically distributed. The effect of the statistically significant difference (also referred to as significant difference) is evaluated by at least one of 4 criteria. These criteria can be subjective where either the effect on clinical outcome is considered or the degree of overlap of the boxplots is compared or it can be objective by comparing the statistically significant difference to the analyser imprecision or the relative change values for the analyte.

C.4 Results

The results are divided into:

1. comparison of different bicarbonate compounds
2. stability
3. comparison of DxC total carbon dioxide (tCO_2) with blood gas analysis
4. reference range
5. functional sensitivity.

All the results are the difference between the measured tCO_2 of the subject's spiked specimen and the subject's native (unspiked) specimen.

C.4.1 Comparison between sodium bicarbonate and ammonium bicarbonate

The tCO_2 of the spiked samples analysed initially ($t = 0$) are depicted in Figure C.3 where the predicted bicarbonate concentrations are on the x -axis, the bicarbonate recovered is on the y -axis and each of the box-and-whisker

plots represents either a NH_4HCO_3 sample or a $NaHCO_3$ sample as stated along the x -axis. The description of the raw data (Table S-1) and the inferential statistics (Table S-2) are provided in Supplemental data S-3.

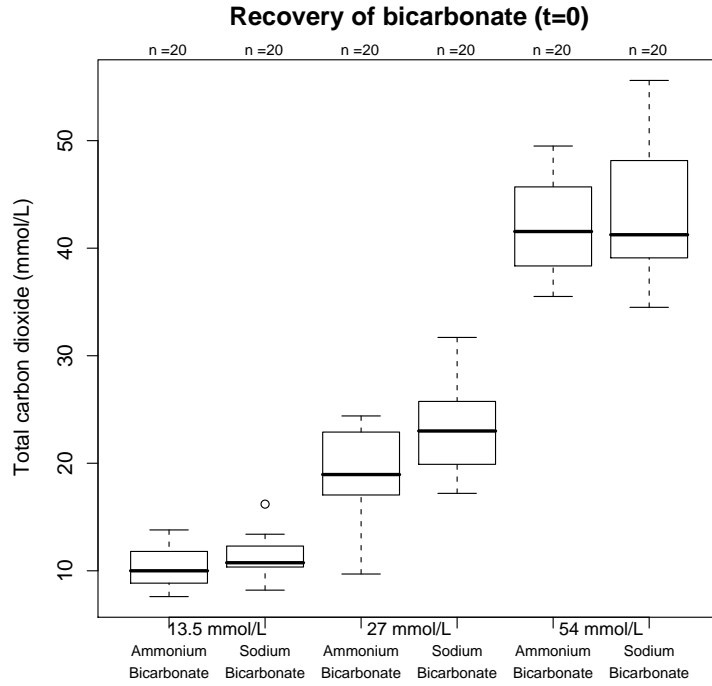


Figure C.3: RECOVERY (IMMEDIATELY AFTER SPIKING) OF BICARBONATE ADDED TO URINE SPECIMENS

The results in Figure C.3 were combined by solution type (NH_4HCO_3 vs $NaHCO_3$) and plotted as a recovery ratio in Figure C.4. The paired student t -test was used to show a statistically significant difference ($p = 7.8 \times 10^{-6}$) between the mean recovery ratio of NH_4HCO_3 and $NaHCO_3$. The recovery of the central 90% of NH_4HCO_3 is in the range 60 – 91%. For $NaHCO_3$ the central 90% recovery is in the range 64 – 103%. Ammonium bicarbonate read 7.5% less (95% $CI = 4.4 - 10\%$).

Bicarbonate recovery was not significantly different between NH_4CO_3 and $NaHCO_3$ (Figure C.3). In the 13.5 $mmol/L$ and 27 $mmol/L$ samples,

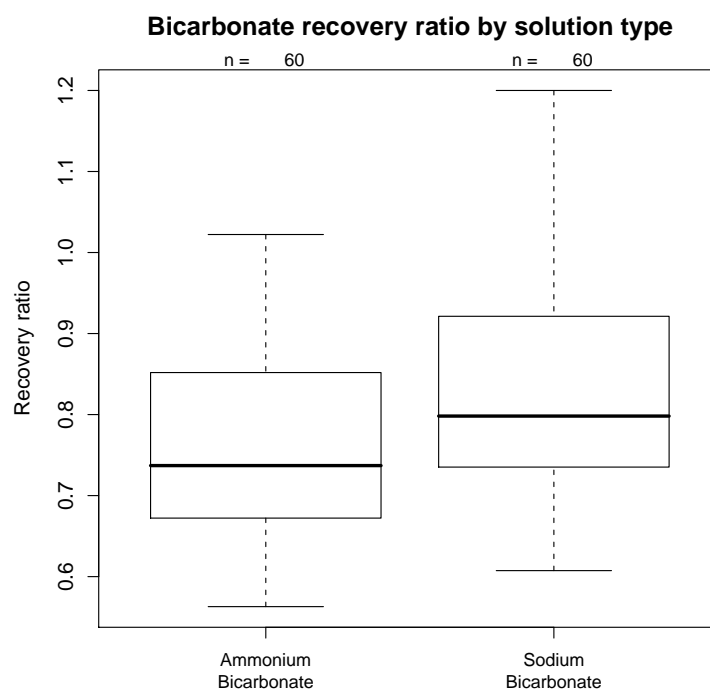


Figure C.4: RECOVERY RATIO OF BICARBONATE BY SOLUTION TYPE (ANALYSIS PERFORMED IMMEDIATELY POST SPIKING)

the $NaHCO_3$ variability appears to be less than that of the NH_4CO_3 sample. The dissolution process was much longer for ammonium bicarbonate and this may account for the variability. The sodium bicarbonate variability of the 54 mmol/L sample was greater than that of the corresponding ammonium bicarbonate sample. This is likely to be due to the dilution of the sodium bicarbonate solution to measure tCO_2 , since it exceeded the upper limit of the measuring range. Furthermore, the sodium bicarbonate solution generally reads a higher tCO_2 than ammonium bicarbonate. Both ammonium bicarbonate and sodium bicarbonate recoveries were considerably lower than the expected 100% (Figure C.4).

C.4.2 Stability experiments

For the NH_4HCO_3 sample, the effect of temperature at 2 h (Figure C.5) and 24 h (Figure C.6) is shown, where each concentration is stratified into initial concentration, storage at room temperature and storage at 4°C.

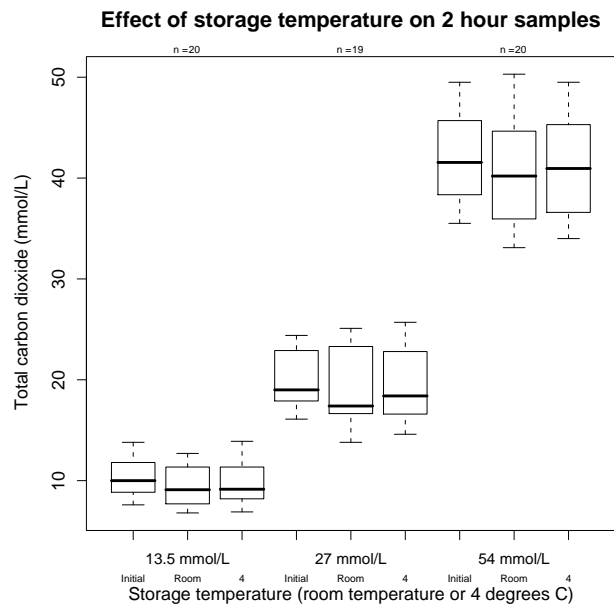


Figure C.5: EFFECT OF TEMPERATURE ON THE STABILITY OF AMMONIUM BICARBONATE AFTER 2 HOURS FOR THE 3 DECISION LEVELS – n: count, room: room temperature, initial: at $t = 0$

No difference exists in measurement whether the analyte is stored at room temperature or at 4°C (Figures C.5 and C.6). The effect of storage time on the NH_4HCO_3 room temperature specimens (Figure C.7) and on the refrigerated specimens (Figure C.8) is shown. Regarding the effect of storage time on recovery, a significant difference is not evident. However, there is increased variability after 24 h (Figures C.7 and C.8).

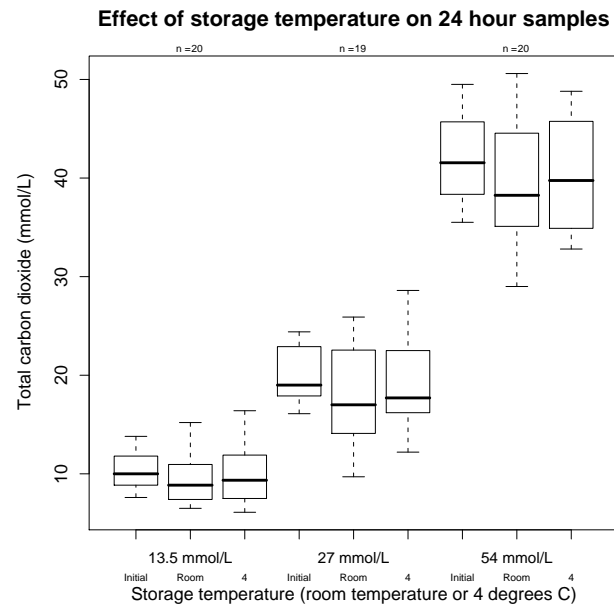


Figure C.6: EFFECT OF TEMPERATURE ON THE STABILITY OF AMMONIUM BICARBONATE AFTER 24 HOURS FOR THE 3 DECISION LEVELS– n: count, room: room temperature, initial: at $t = 0$

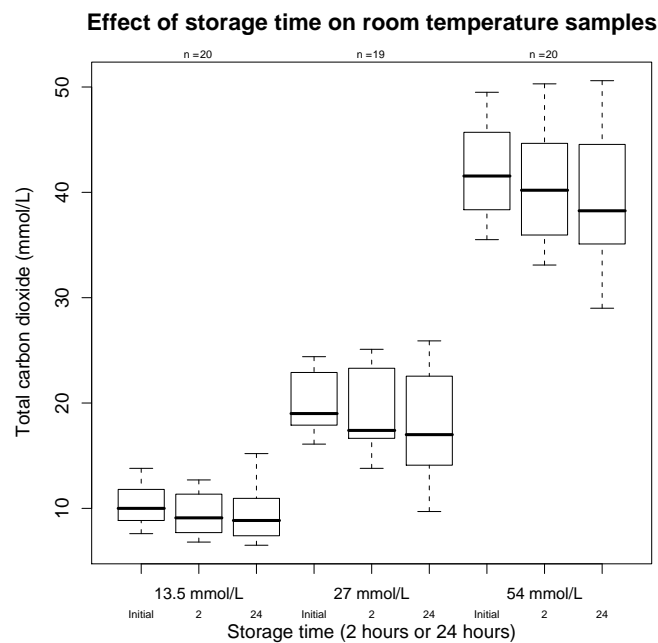


Figure C.7: EFFECT OF STORAGE TIME ON THE STABILITY OF AMMONIUM BICARBONATE STORED AT ROOM TEMPERATURE FOR THE 3 DECISION LEVELS n: count, initial: at $t = 0$

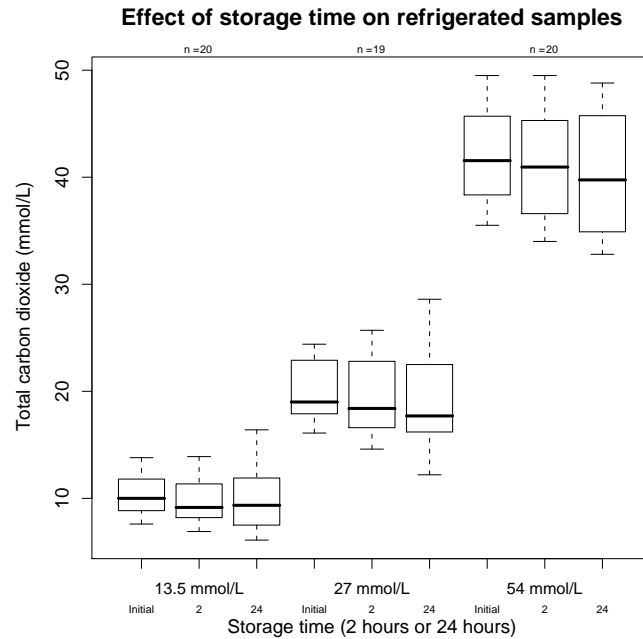


Figure C.8: EFFECT OF STORAGE TIME ON THE STABILITY OF REFRIGERATED AMMONIUM BICARBONATE FOR THE 3 DECISION LEVELS refrigeration temperature: 4°C , n: count, initial: at $t = 0$

The descriptive and inferential statistics for the effect of storage temperature and storage time confirm the above observations and is presented in the supplemental data (Table S-3 and Table S-4, respectively).

C.4.3 Blood gas analysis comparison

The majority of these tests will be conducted in peripheral laboratories which may not have an automated analyser for the measurement of $t\text{CO}_2$ but may have a point-of-care arterial blood gas analyser (ABG). Thus, the 24 h NH_4HCO_3 specimens were analysed on both the DxC and ABG analysers (Figure C.9). ABG $t\text{CO}_2$ rather than HCO_3 was compared to DxC $t\text{CO}_2$ because, from Equation (C-2), the former is the equivalent to the DxC $t\text{CO}_2$.

The recoveries on the DxC and ABG analysers are comparable (Figure C.9 and Supplemental Table S-5). However, the Wilcoxon-test indicates sig-

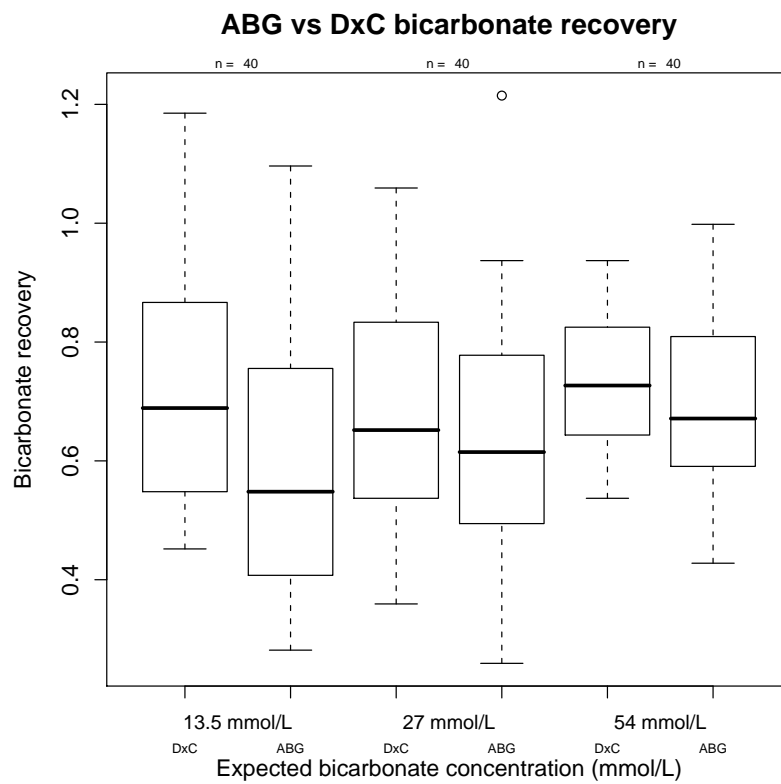


Figure C.9: 24 HOUR AMMONIUM BICARBONATE RECOVERY ON THE DxC AND ABG ANALYSERS – ABG: Bayer RapidLab[®] 348 blood gas analyser, DxC: Beckman-Coulter DxC[®], n: count

nificant differences between the ABG and DxC measurements but the contribution of these differences are not significant (Supplemental Tables S-5 and S-6). The variability in recovery reduces with increasing spiked concentration – the 54 *mmol/L* ammonium bicarbonate solution was rarely analysed in dilution (Figure C.9). This can be accounted for both by the increased variability in the measurement of the lower masses and the relatively greater contribution of innate urinary bicarbonate (that was below the limit of detection (LoD) of 5 *mmol/L*) at the lowest bicarbonate level.

C.4.4 Functional sensitivity

The functional sensitivity was interpolated by using the method of least squares to fit a power function ($y = ax^b + c$) to the precision data for the initial ammonium bicarbonate measurements. The power function was selected because it approaches an asymptote at infinity and approaches infinity at zero – analogous to the functional sensitivity. The coefficient of variation (CV) and standard deviation (SD) were calculated from the measured bicarbonate (Table C.1).

		Ammonium bicarbonate <i>mmol/L</i>			Sodium bicarbonate <i>mmol/L</i>		
Expect	n	μ	SD	CV (%)	μ	SD	CV (%)
13.5	20	10.1	1.88	18.6	11.2	1.87	16.6
27	20	20.0	2.87	14.3	23.0	3.94	17.0
54	20	41.7	4.09	9.81	43.4	5.93	13.7

Table C.1: INTRA-ASSAY PRECISION DATA FOR BICARBONATE: COMPARISON BETWEEN $NaHCO_3$ AND NH_4HCO_3 – n: count, SD: standard deviation, CV: coefficient of variation, Expect: expected bicarbonate concentration (*mmol/L*), μ : *mean*

Equation (C-3) is the formula relating CV to the mean of the measured bicarbonate. Consequently, the CV for urinary bicarbonate (as a function of

measured bicarbonate) is

$$CV(\%) = 54\langle NH_4HCO_3 \rangle^{-0.453}, R^2 = 0.99 \quad (C-3)$$

where $\langle NH_4HCO_3 \rangle$ is the mean of the measured bicarbonate when NH_4HCO_3 is added. Consequently, the functional sensitivity is 8.95 mmol/L . The formula for the CV based on expected bicarbonate is given by

$$CV(\%) = 63.4 (E(NH_4HCO_3))^{-0.463}, R^2 = 0.99 \quad (C-4)$$

where $E(NH_4HCO_3)$ is the expected bicarbonate when NH_4HCO_3 is added. The functional sensitivity for the expected bicarbonate is 12 mmol/L .

Similarly, the CV based on the measured bicarbonate when using sodium bicarbonate is

$$CV(\%) = 24.4\langle NaHCO_3 \rangle^{-0.142}, R^2 = 0.62 \quad (C-5)$$

where $\langle NaHCO_3 \rangle$ is the mean of the measured bicarbonate when $NaHCO_3$ is added. Consequently, the functional sensitivity is 4.1 mmol/L . The formula for the functional sensitivity based on expected bicarbonate is given by

$$CV(\%) = 25.2 (E(NaHCO_3))^{-0.143}, R^2 = 0.66 \quad (C-6)$$

where $E(NaHCO_3)$ is the expected bicarbonate when $NaHCO_3$ is added. The functional sensitivity for the expected bicarbonate is 5.0 mmol/L , and lower values should be reported as $< 5 \text{ mmol/L}$.

C.4.5 Recovery difference experiments

The predicted SD for the difference between the 27 mmol/L and 13.5 mmol/L ammonium bicarbonate standards is 3.43 mmol/L (Table C.1). The predicted CV (based on the mean of the differences) is 34.5%. Similarly the predicted SD for the difference between the 54 mmol/L and 13.5 mmol/L ammonium bicarbonate standards is 4.5 mmol/L , with a CV of 14.2%[18].

The measured CV of the difference between the 27 mmol/L and 13.5 mmol/L standards for ammonium bicarbonate is 16%. The measured CV for the difference between the 54 mmol/L and the 13.5 mmol/L standards for ammonium bicarbonate is 8.2%.

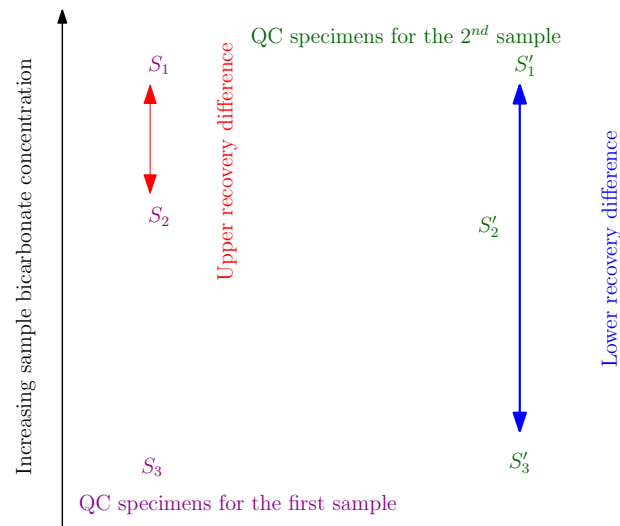


Figure C.10: THE MEASUREMENT OF DIFFERENCE IN RECOVERY: the measurements are performed in duplicate – first on sample 1 then on sample 2. S_x specimen number x in sample 1 where $x = 1, x = 2$ and $x = 3$ represent the high, middle and low controls, respectively. S'_x specimen number x in sample 2

The reduction in variability when measuring the difference in bicarbonate standards as compared to predicted variability (Section C.4.5) supports the conclusion that it is the innate, unmeasured urinary bicarbonate that increases the variability. By measuring the difference in recovery (Figure C.10),

the uncertainty associated with the unmeasured, innate urinary bicarbonate is eliminated (Supplemental data S-1).

C.4.6 Reference range

Twenty-five specimens were analysed after adjusting the DxC analyser to report results greater than 3 mmol/L – allowing results below the LoD (of 5 mmol/L) to be reported. Reporting results below the LoD is unacceptable for a single result, but can be performed for a sample consisting of several specimens. Of the 25 results, 12 were $< 3 \text{ mmol/L}$ and 13 were recordable ($\geq 3 \text{ mmol/L}$). Thus the median (M) result is 3 mmol/L . Twelve of the results were mirrored (thus a symmetric distribution is assumed) about M thus accounting for the 12 unmeasurable specimens. Furthermore, given the imposed symmetry, the mean (μ) equals M . Of the resultant 25 results, 2 were rejected as outliers – the highest urine bicarbonate produced a negative value when mirrored and thus both the highest value and its mirror were rejected. The calculation of the reference range is depicted in Figure C.11. The absence of normality could not be confirmed either by Shapiro-Wilk test or QQ plot.

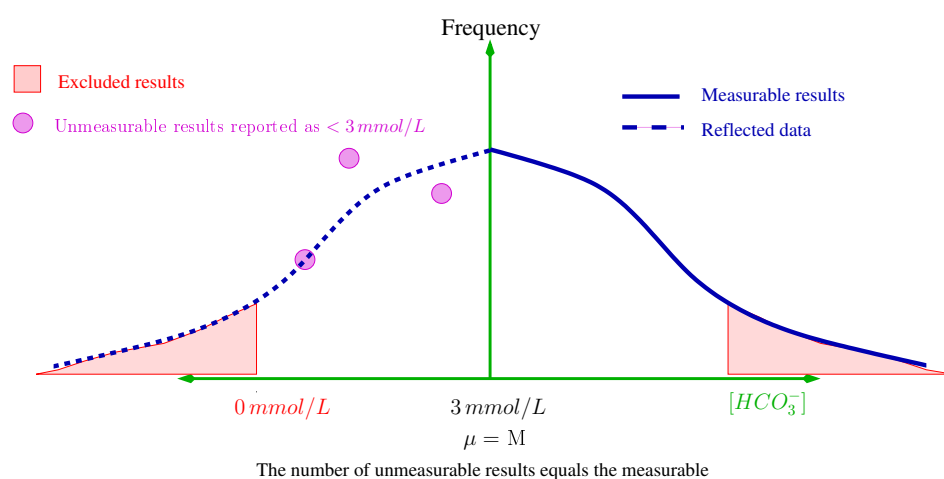


Figure C.11: REFERENCE RANGE DETERMINATION – μ : mean, M : median

The mean urine bicarbonate was 3 mmol/L with a SD of 0.5 mmol/L . The reference range for urine bicarbonate was thus calculated as $2\text{--}4\text{ mmol/L}$. The 90% confidence interval of the upper limit of the reference range is thus $3.8\text{--}4.2\text{ mmol/L}$. A reference range for comparison could not be located.

The reference range has been calculated on a small sample with the assumption of symmetry. The correct method would be to use non-parametric analysis (requiring considerably more specimens) where results below the LoD are assigned the value 0. One would then only need to calculate the 97.5th centile and the 90% confidence interval for that centile. A reference range is required for validation before the urine bicarbonate test can be implemented.

C.4.7 Summary of urinary bicarbonate analysis validation

The analysis of substrates indicates that, analytically, the use of NaHCO_3 as a quality control (QC) material may be superior to NH_4HCO_3 since the recovery and precision are greater (Table S-2 and Figures C.3 and C.4) but the differences are negligible (Table S-2) when compared to the measurement precision (Table C.1) and reference change values (Table C.3). The storage temperature has little effect on the recovery of bicarbonate in samples stored in microcentrifuge tubes (Figures C.5 and C.6). It is proposed that samples should be analysed within 2 h because of the improved precision compared to samples stored for 24 h (Figures C.7 and C.8).

Although a statistically significant difference exists between the analyser-specific results of the ABG $t\text{CO}_2$ and the DxC $t\text{CO}_2$ (Table S-6), the effect of the difference is insignificant (Figure C.9 and Tables S-6, C.1 and C.3). One

can thus conclude that the results of the ABG tCO_2 are comparable to the DxC tCO_2 . On the DxC instrument, the functional sensitivity is 5 mmol/L and the reference range is $2 - 4\text{ mmol/L}$.

Finally, by using the difference in recovery, rather than actual recovery, better precision is achieved (Section C.4.5). Thus an ILC program should be based on difference in recovery.

C.5 Proficiency testing design

Sodium bicarbonate was selected as the material for the ILC program since it has reduced variability, lower cost, easier dissolution and is less noxious. Furthermore, this study and a study of the effect of different sodium concentrations on the pK_a of carbonic acid[13] indicate that the use of sodium bicarbonate does not affect tCO_2 measurement. To avoid the imprecision of weighing small quantities of sodium bicarbonate, it is proposed that the minimum concentration spiked for the PT program should be 10 mmol/L . An alternative would be to use large volumes of urine such that larger masses of sodium bicarbonate can be weighed. To avoid the increased variability caused by the need for post-analytical dilution of high HCO_3^- samples, the maximum concentration spiked should not exceed 40 mmol/L .

To negate the problem of innate urinary bicarbonate that is below the LoD, it is proposed that the difference in recovery should be measured. The proof that the difference in recovery is independent of initial concentration is provided in Supplemental data S-1. In general, two measurements increase the uncertainty of measurement[18] relative to a single measurement, but not in this case (Section C.4.5). Lastly, since the $NaHCO_3$ will be weighed, it would be preferable if only one mass is weighed for all three concentrations

and that the final concentration is obtained by varying the volume of urine added.

The CLSI[12] provides several options for alternate assessment procedures (AAPs) in the absence of a PT program. Eleven options (seven quantitative) are provided that are summarised in Table C.2 by the minimum number of laboratories for optimal implementation and the minimum number of routine patient samples required for a given time period. It is not anticipated

	Many laboratories	At least 1 laboratory
Many tests	All options	Average of patient results Continuous re-evaluation of reference range Clinical correlation studies
Few tests	Split sample/specimen Manufacturer calibrant IQC data analysis	Audit sample procedure Government/university ILC

Table C.2: COMPARISON OF ALTERNATIVE ASSESSMENT PROCEDURES FOR PROFICIENCY TESTING – ILC: interlaboratory comparison, IQC: internal quality control

that many tests will be performed initially and the vast majority of patient samples will have undetectable urine bicarbonate – below the LoD (Section C.4.6). Thus only the AAPs that require few routine samples are potential solutions. To avoid matrix effects, the only viable options are split sample/specimen testing and the audit sample procedure. Split sample testing requires that a single sample should be divided into aliquots and the individual aliquots are tested by several laboratories. Audit sample testing involves testing of stored aliquots of the same biological sample repeatedly over time on the same assay system. Initially it is anticipated that the audit sample procedure will have greater utility until sufficient laboratories participate to allow split sample/specimen result interpretation. It is recognised that the stability of bicarbonate-spiked sample, both during transport and storage, cannot be guaranteed and that lyophilisation is not an option.

Variants of split-specimen testing and audit-sample testing[12] are proposed as potential AAPs. It is proposed that fixed quantities of sodium bicarbonate for spiking should be stored and transported (analogous to lyophilised samples). The storage vessels will be 2 ml (2.2 ml), 1.5 ml (1.65 ml) and 0.5 ml (0.55 ml) microcentrifuge tubes. To each of the tubes add 2 mg of sodium bicarbonate ($\pm 0.2\text{ mg}$). Document the amount added. When 2 ml , 1.5 ml and 0.5 ml of urine are added to their respective microcentrifuge tubes, the concentrations should be 11.9 mmol/L , 15.87 mmol/L and 47.6 mmol/L respectively. Should the amount added differ from the 2 mg by δx (a small change), the amount of urine that needs to be added is adjusted by the factor $\delta x/2 + 1$. Although it is recognised that $E(\text{HCO}_3^-)$ for the lower standard is less than the functional sensitivity based on $E(\text{NH}_4\text{HCO}_3)$ in Section C.4.4, given that the recovery for NaHCO_3 is greater than that for NH_4HCO_3 (Figure C.4), it is anticipated that the imprecision at $E(\text{NaHCO}_3) = 11.9\text{ mmol/L}$ will be less than the functional sensitivity based on $E(\text{NH}_4\text{HCO}_3)$ (Equations (C-4) and (C-6)). This is a necessary compromise to ensure that the masses can be measured reproducibly whilst avoiding sample dilution which appears to increase variability (Figure C.3).

Duplicate samples, each consisting of the above three specimen standards, are sent to laboratories for testing – with the amount of urine to be added documented. The bicarbonate analysis is conducted within 1 h of adding the urine. The purpose of two samples is to test for error in the bicarbonate added. The laboratories can use urine from any subject and pipette the appropriate amount of urine, analyse and return the results together with the details of the instrument used. The maximum value by which corresponding standards in each of the samples may differ (as calculated from the functional sensitivity formula in Section C.4.4 and the reference change value[18]

where the biological variation is ignored) at each of the levels is summarised in Table C.3. It is accepted that no error in weighing the $NaHCO_3$ has

Bicarbonate added (<i>mmol/L</i>)	Maximum difference (<i>mmol/L</i>)
11.9	6
15.9	7
47.6	19

Table C.3: MAXIMUM ALLOWABLE DIFFERENCE BETWEEN DUPLICATES IN PROFICIENCY TESTING PROGRAM

occurred if the maximum allowable difference between duplicated standards is not exceeded. Initially ILC should be conducted monthly. The monthly ILC is defined as a QC cycle here.

For the modified split specimen procedure, the difference between the 47.6 *mmol/L* and 11.9 *mmol/L* specimens and the difference between the 15.87 *mmol/L* and 11.9 *mmol/L* specimens are plotted on a Youden[19] plot if and only if the error in the measure of the duplicated standard results is within the acceptable range. The requirement that the corresponding specimen results between The Youden plots are interpreted as for any PT program[20] with limits arbitrarily set to 2SD for all the laboratory results or to the predicted 2SD.

For the audit sample procedure, the standard deviation index (SDI) is plotted with respect to time and only rejected if a result falls outside of 2 SDI. The SDI is measured relative to the group SD or the predicted SD.

C.6 Prototype results

Laboratory A determined the total CO_2 on a Bayer[®] Rapid Lab ABG analyser. Laboratory B determined the tCO_2 on a Beckman-Coulter[®] DxC analyser. Laboratory C performed the tCO_2 on both a Beckman-Coulter[®] CX7

(using the same acidification method as the DxC) and on Roche® COBAS B221 ABG analyser.

Samples were sent to the three laboratories, thrice during one month. The tCO_2 differences (maximum less minimum and middle less minimum) were charted on a Youden plot (Figure C.12). Youden plots should only chart one point per laboratory at a time. Here, the results for all three times have been plotted. Laboratory C was unable to measure the high standard on the third run. Due to the small sample size and the repetition of laboratories, the

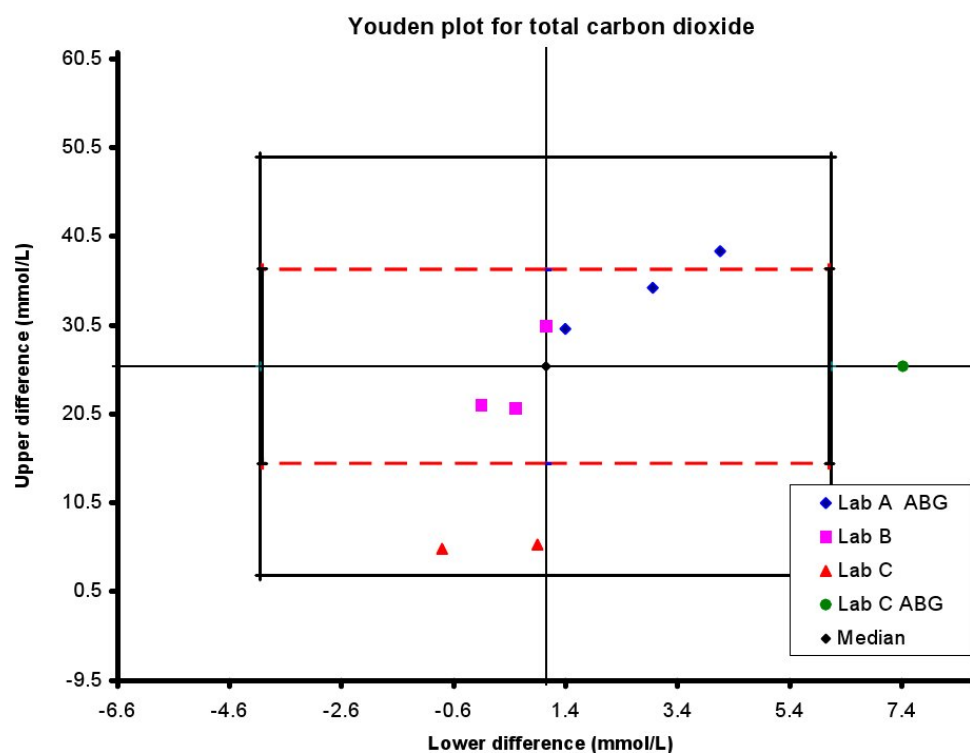


Figure C.12: TOTAL CO_2 YOUDEN PLOT: The solid black box represents two standard deviations for all the samples in the plot about the median difference. The dashed red box represents double the predicted standard deviation about the median difference. ABG: arterial blood gas analyser

median is affected by outliers. This is most evident with the lower recovery difference (Figure C.12). Laboratory C failed on the one occasion that tCO_2 was performed on the ABG instrument. Using the more stringent criteria for

acceptable results, by setting the limits at double the predicted SD (dashed red box), Laboratory C failed consistently and Laboratory A failed once. Laboratories A and B have proportional error, while laboratory C's error is not proportional.

The EQA was also evaluated using the SDI for the three laboratories. The y-axis represents the SDI for the upper difference and is relative to the sample's upper difference SD at that sample number (C.13). The dashed red lines represent SDI two units where the SD is the predicted SD.

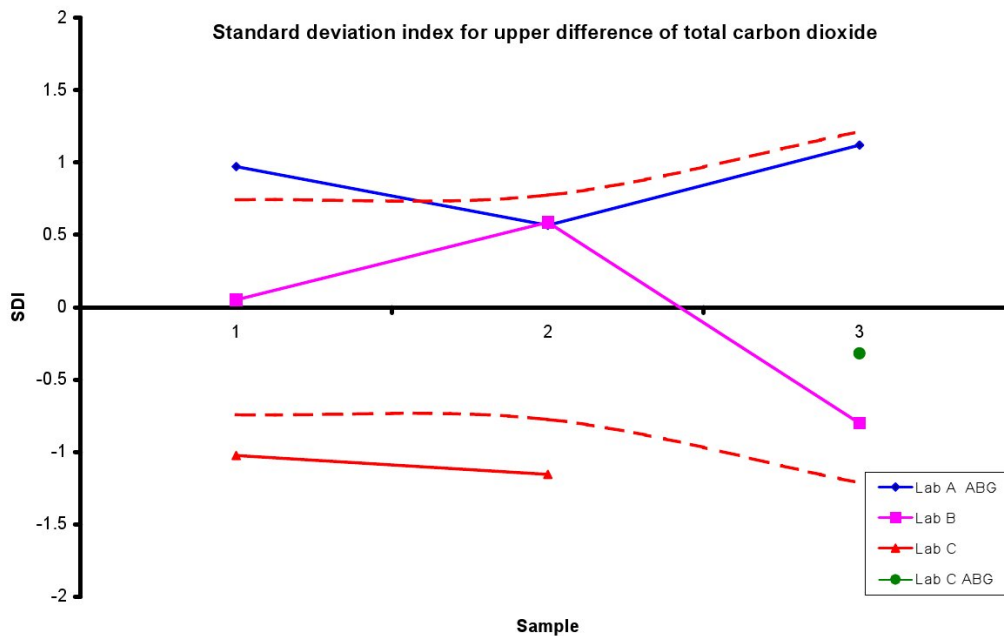


Figure C.13: TOTAL CO_2 UPPER DIFFERENCE SDI PLOT: The dashed red lines represent two standard deviation index units (based on predicted standard deviation). ABG: arterial blood gas analyser

The SDIs for the lower differences were acceptable for all the laboratories (Figure C.14). The dashed red lines represent two SDI units when normalised to the predicted SD for the lower recovery difference.

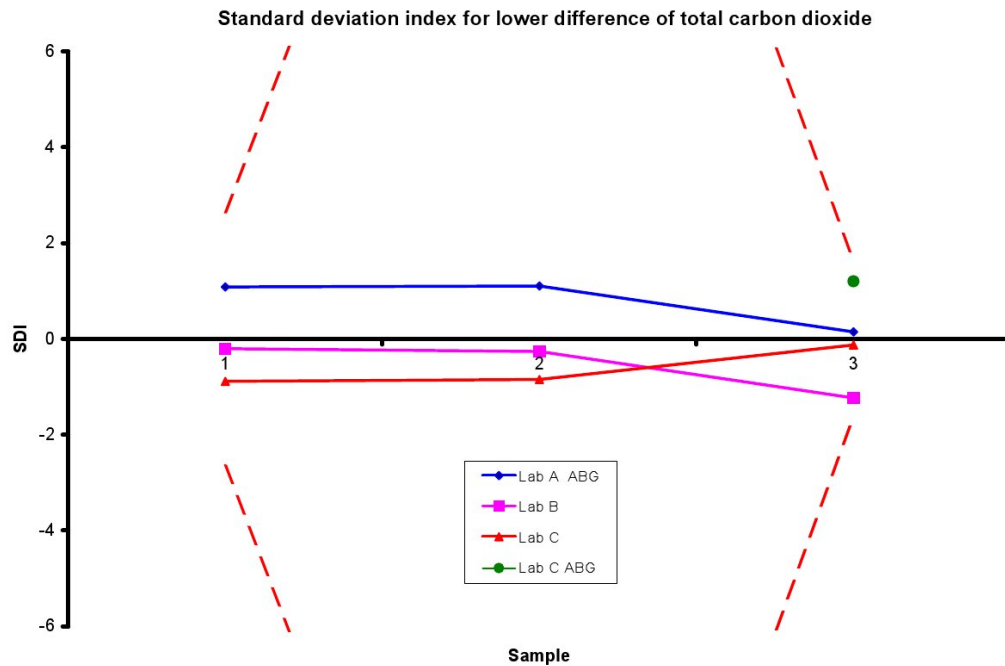


Figure C.14: TOTAL CO_2 LOWER DIFFERENCE SDI PLOT: The dashed red lines represent two standard deviation index units (based on predicted standard deviation). ABG: arterial blood gas analyser

The recovery of the upper difference was acceptable for all but one laboratory (Figure C.15). The dashed red lines represent the acceptable recovery range based on Section C.4.5. Laboratory C under-recovered significantly. Similarly, in the lower difference recovery (Figure C.16), the dashed red lines represent the predicted acceptable range of recovery. Again, the wide range is indicative of the imprecision at these analyte levels.

C.7 Discussion of pilot study of the ILC program

This pilot study of the ILC program has highlighted a need for a proficiency testing program for urine bicarbonate because it is evident that laboratory C is performing suboptimally.

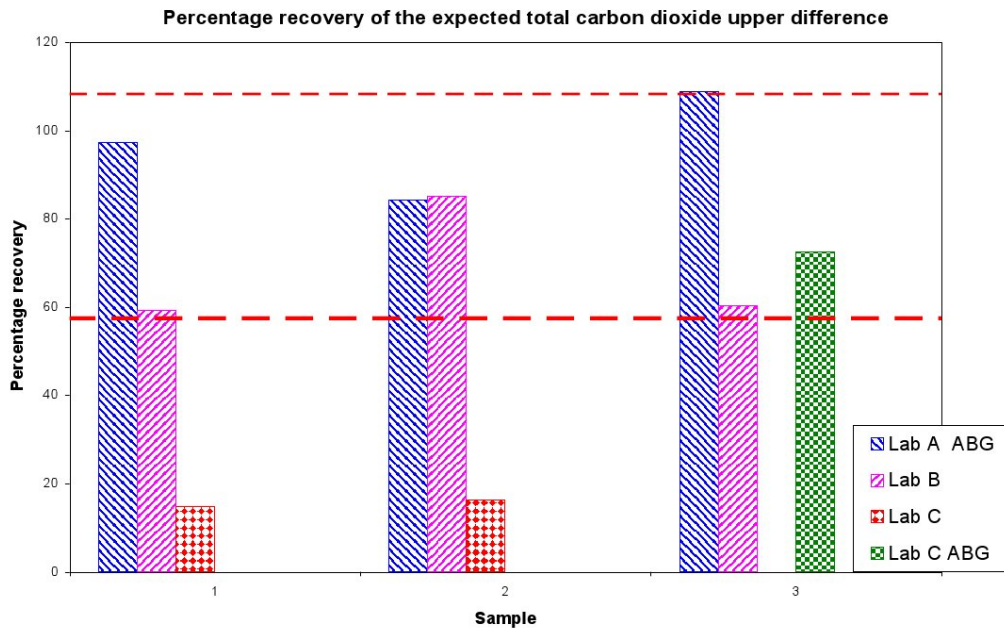


Figure C.15: TOTAL CO_2 UPPER DIFFERENCE RECOVERY: The dashed red lines represent the predicted range of recovery results. ABG: arterial blood gas analyser

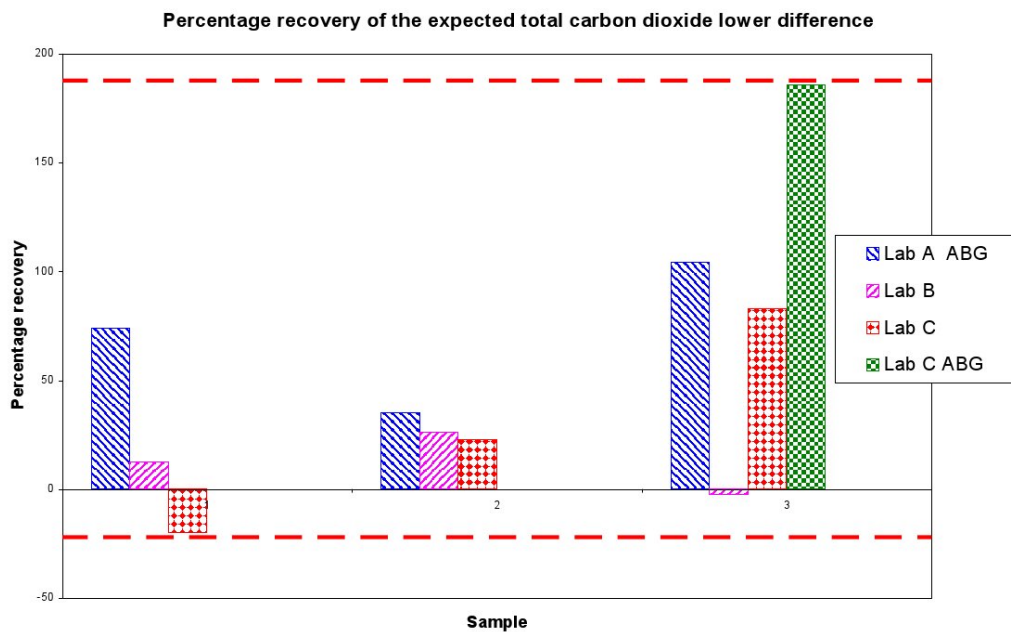


Figure C.16: TOTAL CO_2 LOWER DIFFERENCE RECOVERY: The dashed red lines represent the predicted range of recovery results. ABG: arterial blood gas analyser

Despite the limited number of samples and the pooling of all the laboratory results, the Youden plot (Figure C.12) detected flaws in laboratory C's ABG analyser measurements and in its Beckman-Coulter tCO_2 at the more stringent criteria of predicted SD. Laboratory A appears flawed for one run, but this is a consequence of the median being distorted by the results of laboratory C. The distortion of the median is due to the low specimen number and using multiple results from the same laboratories. Laboratory A is performing within limits, as evidenced by the recovery experiments (Figure C.15).

The flaw in laboratory C's results is highlighted by the upper difference SDI plot (Figure C.13). Importantly, the SDI plots (Figures C.13 and C.14) do not suggest a flaw in laboratory C's PT results – evident in the Youden plot (Figure C.12). Thus, the SDI plots and the Youden plot are complementary.

The inherent flaw in both the SDI and Youden plots is that the sample median does not necessarily reflect the true value. The recovery experiments are complementary to the SDI and Youden plots by being relative to a higher (here gravimetric) standard. Thus, the upper difference recovery plot (Figure C.15) provides information on the quality of the recovery.

The wide acceptable limits for the lower recovery difference (Figures C.14 and C.16) diminishes the ability of this metric to detect a flaw. The origin of the wide limits is the reduced precision at the lower recoveries used to determine the recovery difference. The effect of the variability on the CV of the lower recovery difference is compounded by the small difference between the 11.9 mmol/L and 15.9 mmol/L standards.

A marginal cost analysis was performed in order to ensure that the ILC program is cost effective (Supplemental S-2).

C.8 Conclusion

An interlaboratory comparison program has been designed for an unstable analyte (HCO_3^-) that is only infrequently measured in a few laboratories. The program relies on the measurement of the difference in recovery of total CO_2 in urine samples spiked with $NaHCO_3$. Matrix effects and instability associated with transport and refrigeration are minimised by adding fresh human urine to the dry reagent within 1 *h* of analysis.

The procedure can be generalised to certain classes of unstable analytes that are only infrequently measured at a few laboratories (eg. acetoacetate and pyruvate).

The design of the program was based on a series of experiments comparing ammonium bicarbonate to sodium bicarbonate (in urine) on two analysers. Sodium bicarbonate was a superior analyte in terms of analytical performance and cost. A reference range was established based on 23 specimens and although the sample size may be sub-optimal, the utility of more detailed reference range analysis is debatable, given that the range is less than the functional sensitivity. The functional sensitivity proved to be more useful than the limit of detection in assessing the analytical performance of the assay. The limit of detection and the limit of blank could not be determined because the analyser could not be set to read to this level. Values less than 5 mmol/L are reported as $< 5\text{ mmol/L}$. The analyser can be adjusted to read to $\geq 3\text{ mmol/L}$.

The initial implementation of the AAP has proven to be successful and should continue to be implemented in the Western Cape. The ABG instrument performed better than the DxC analyser, in terms of both recovery and variability, even though tCO_2 is measured indirectly by the ABG analyser and directly by the DxC. This is likely to be due to the reduced delay in processing on the ABG, such that the specimen is exposed to air for a shorter period.

The cost of running the ILC (Supplemental S-2) is $R4,64$ per QC cycle per laboratory where a QC cycle comprises 6 recovery of bicarbonate tests. It is proposed that one QC cycle should occur each month. The marginal cost[21] of performing the ILC on the Beckman-Coulter DxC[®] is $R17,64$ (ILC plus reagent costs). The marginal cost of performing the ILC on an arterial blood gas analyser is only the cost of the ILC program ($R4,64$) because of the nature of the lease agreement.

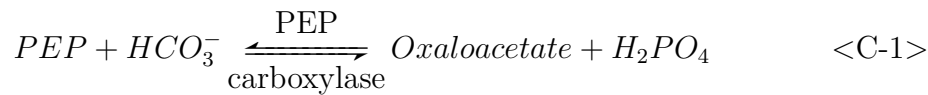
It is proposed that both the urine bicarbonate samples and QC samples be analysed on the arterial blood gas analyser, since it is more cost effective and the urine is exposed to the atmosphere for a shorter period of time.

The value of the lower recovery difference may be improved by increasing the concentration of the middle recovery level (15.9 mmol/L). Increasing the middle recovery level to 23.9 mmol/L by reducing the volume of urine added to the 1.5 ml microcentrifuge tube to 1.0 ml , should reduce the acceptable limits of the lower recovery difference (Figures C.14 and C.16).

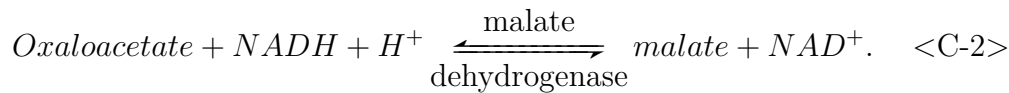
Clinically, the design and implementation of the EQA program will facilitate multicenter trials, allow comparison of laboratory results and allow epidemiological studies on the prevalence of RTA in various groups.

This dissertation constructed a PT program and evaluated tCO_2 determined by acidification of all bicarbonate species to CO_2 and then measuring with a CO_2 electrode on an automated analyser. A second method determined pH and pCO_2 using H^+ and CO_2 electrodes respectively, and then calculating tCO_2 on an ABG analyser. The latter appears to be more precise. It is believed that the improved precision of the ABG analyser is because the sample is exposed to air for a shorter period of time – HCO_3^- is volatile.

It is tempting to think that an enzymatic method like



where PEP is phosphoenolpyruvate and the resultant oxaloacetate is measured spectrophotometrically[22] using the reaction



However, based on the improved precision of the ABG (presumed due to less air exposure), this dissertation predicts that the precision of the enzymatic method, on an automated analyser, will be compromised by the delay in processing during which the specimen will be exposed to air.

C.9 Acknowledgments

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Supplementary data

S-1 Recovery difference: proof of concept

Let the initial (before spiking) HCO_3^- concentration of a random urine (U_x) be c . Let two containers have volumes V_1 and V_2 , respectively. If, to each container, a volume of urine (U_x) is added, the amount of bicarbonate added to each container is

$$n_1 = c \times V_1 \quad (\text{S-1})$$

$$n_2 = c \times V_2 \quad (\text{S-2})$$

Let the amount spiked into each container be N_1 and N_2 , respectively. Thus the amount of HCO_3^- in each container is $n_1 + N_1$ and $n_2 + N_2$, respectively. For container 1, the concentration is then given (upon substitution of (S-1))

$$c_1 = \frac{n_1 + N_1}{V_1} \quad \Longleftrightarrow \quad c_1 = c + \frac{N_1}{V_1}. \quad (\text{S-3})$$

Similarly, for the second container,

$$c_2 = \frac{n_2 + N_2}{V_2} \quad \Longleftrightarrow \quad c_2 = c + \frac{N_2}{V_2}. \quad (\text{S-4})$$

Thus the difference in the measured concentrations, (S-3) - (S-4), is

$$c_1 - c_2 = c - c + \frac{N_1}{V_1} - \frac{N_2}{V_2} = \frac{N_1}{V_1} - \frac{N_2}{V_2} \quad (\text{S-5})$$

which is independent of the original urine concentration c . For this dissertation, the difference in recovery is defined by the left-hand-side of (S-5) or $c_1 - c_2$.

S-2 Proficiency testing costing

Initially this ILC program is intended to be implemented at the tertiary academic hospitals of the Western Cape – three NHLS laboratories.

Given the low test volume, the ILC program will be implemented from one of the above laboratories – whichever has the most spare capacity. The indirect costs will therefore be borne by the routine diagnostic laboratory. No capital expenditure is required. Several of the direct costs are also negated by existing infrastructure. In particular, a transport system exists between these laboratories, (given that the program will be assigned to the laboratory with excess capacity) no additional labour costs are incurred (25 minutes per laboratory are required to prepare the QC samples), there are no special storage or transport requirements for sodium bicarbonate and results will be transmitted electronically.

Thus the only direct costs to be considered are direct materials (sodium bicarbonate and microcentrifuge tubes) and consumables – paper. All costs are calculated per institution per QC cycle (once a month) with a 10% margin of error included. Each institution uses 2 mg of sodium bicarbonate per test and there are six tests in a QC cycle. Thus, per institution, up to 13.2 mg of sodium bicarbonate is used costing R2,11. The two 0.6 ml microcentrifuge tubes used by each institution cost R0,67, the two 1.5 ml microcentrifuge tubes collectively cost R0,68 and the two 2.0 ml microcentrifuge tubes cost R0,86. Thus the direct materials per institution per QC cycle is R4,32. The

two pages (to be used as hardcopy) cost R0,32. Thus the total cost is R4,64.

Note that the cost of performing the test is not included – this cost is not included when paying for an EQA program. The cost of performing a test is R35,39 (the price that the NHLS would charge a client). Six tests are performed per institution per QC cycle thus totaling R212,34 per institution per QC cycle. Each set of three tests results in two recovery difference levels. The cost (to the institution) of printing the results for its own record keeping is R0,32.

Thus the cost to subscribe to the ILC (including samples and reports) is R4,64 per cycle per institution. The charge to perform the test is R212,66. Thus the total cost to the laboratory is R217,30 per QC cycle.

It should be noted that cost of performing the test includes both direct and indirect costs that should be negated as they are borne by the routine diagnostic tests. Negating the direct and indirect costs that are borne by the routine diagnostic tests, the marginal cost^[1] of performing the ILC on a Beckman-Coulter DxC[®] (ILC cost plus reagents for tCO₂) is R17,64 per QC cycle where the reagents are buffer (R8,00 for 6 tests), reference material (R2,10 for 6 tests), acid reagent (R1,33 for 6 tests), pipette tips (R0,11 for 1), transfer pipette (R0,63 for 6) and cups (R0,81 for 6).

The lease agreements for the ABG analysers include a fixed number of tests per month that is in excess of utilisation. Thus the marginal cost of performing the ILC on these analysers is R0,00. Consequently (for a laboratory using an ABG analyser) the marginal cost of the ILC per laboratory per QC cycle is only the cost of participating in the ILC program which is R4,64 per laboratory per QC cycle.

Thus for 12 QC cycles per year, the marginal cost of urine bicarbonate QC on the DxC is R211,68 per year and for an ABG the marginal cost is R55,68 per annum.

S-3 Tabular summaries of raw data

The description of the ammonium bicarbonate and sodium bicarbonate measurements at the three HCO_3^- decision levels (13.5, 27 and 54 mmol/L) indicates that the measurements on the 2 analysers are comparable (Table S-1). The table shows the count, mean, median, 5th and 95th percentiles of the bicarbonate concentration for the two compounds ($NaHCO_3$ and NH_4HCO_3). The p value for the Shapiro-Wilk test of normality indicates that normality cannot be excluded (Table S-1).

Decision level	Substrate	n	mean	median	5 th	95 th	p
13.5 mmol/L	$NaHCO_3$	20	11.3	10.8	8.3	13.5	0.22
	NH_4CO_3	20	10.2	10	7.7	13.1	0.37
27 mmol/L	$NaHCO_3$	20	23.1	23	17.8	30.5	0.48
	NH_4CO_3	20	19.5	19.0	15.8	24.0	0.08
54 mmol/L	$NaHCO_3$	20	43.0	41.3	35.4	54.3	0.09
	NH_4CO_3	20	42.1	41.6	37.0	47.6	0.43

Table S-1: DESCRIPTION OF NH_4CO_3 VS $NaHCO_3$ HCO_3^- CONCENTRATION COMPARISON DATA – p : p -value of the Shapiro-Wilk test for absence of normality ($p \leq 0.05$ is significant), n : count

The inferential statistics comparing the HCO_3^- concentration of $NaHCO_3$ and NH_4CO_3 (using the paired student t -test) indicates that statistically significant differences exist (Table S-2). The p value in Table S-2 indicates the statistical significance of the t -test comparing the $NaHCO_3$ and NH_4CO_3 HCO_3^- concentration by decision level. The difference column refers to the mean of difference – an indication of the effect (or contribution) of the difference between the measured values for HCO_3^- when using $NaHCO_3$ versus

NH_4HCO_3 .

Decision level	n	p	difference	95% CI
13.5 mmol/L	20	0.02	-1.1 mmol/L	[-1.95, -0.22]
27 mmol/L	20	3×10^{-4}	-3.62 mmol/L	[-5.35, -1.89]
54 mmol/L	20	0.43	-0.93 mmol/L	[-3.33, 1.47]

Table S-2: SUMMARY OF PAIRED STUDENT t -TEST COMPARISON BETWEEN NH_4CO_3 AND $NaHCO_3$ BICARBONATE CONCENTRATIONS BY DECISION LEVEL – p : p -value of the paired student t -test ($p \leq 0.05$ is significant), n : count, difference is the mean of the differences between the NH_4HCO_3 and $NaHCO_3$ results and the 95% CI is the 95% confidence interval for the mean of the differences

The raw data were used to construct Figure C.3 in Section C.4.1. Of note, although a statistically significant difference exists between the HCO_3^- concentrations determined using $NaHCO_3$ versus NH_4CO_3 as substrate for each of the decision levels (Table S-2), the contribution of the difference is negligible when compared to the imprecision of the measurement and the reference change values (Tables C.1 and C.3, respectively).

The descriptive and inferential data for the effect of temperature on the specimens stored for 2 h and 24 h indicate that statistically significant differences due to storage temperature do not exist (Table S-3). Increasing sample size may show statistically significant differences but should such an effect exist, it would be negligible compared to instrument imprecision and the reference change values (Tables C.1 and C.3, respectively). In Table S-3 the results are stratified by decision level. The p value is the result of the one-way analysis of variance (ANOVA). ANOVA was performed because normality could not be excluded on the Shapiro-Wilk test.

Similarly, storage time did not have a statistically significant effect on HCO_3^- recovery (Table S-4). When the differences in recovery due to storage time are compared to measurement uncertainty and reference change values (Tables C.1 and C.3), there appears to be no value to increasing sam-

Time	Temp	Bicarbonate concentration (mmol/L)					
		13.5	95% CI	27	95% CI	54	95% CI
2 h	Initial	10.2	[9.4, 11.0]	20.0	[18.7, 21.4]	42.1	[40.3, 43.9]
	RT	9.5	[8.6, 10.4]	19.4	[17.7, 21.1]	40.8	[38.5, 43.1]
	4°C	9.7	[8.8, 10.6]	19.4	[17.7, 21.1]	41.1	[39.0, 43.1]
	<i>p</i>	0.50			0.65		
24 h	Initial	10.2	[9.4, 11.0]	20.0	[18.7, 21.4]	42.1	[40.3, 43.9]
	RT	9.4	[8.4, 10.5]	18.0	[15.8, 20.1]	39.8	[37.2, 42.4]
	4°C	10.1	[8.8, 11.4]	19.0	[17.0, 21.0]	40.3	[37.8, 42.7]
	<i>p</i>	0.56			0.36		

Table S-3: DESCRIPTIVE AND INFERENTIAL DATA FOR THE EFFECT OF STORAGE TEMPERATURE ON BICARBONATE RECOVERY STRATIFIED BY DECISION LIMIT – concentrations are in *mmol/L*, *p*: *p*-value of one-way ANOVA ($p \leq 0.05$ is significant), RT: room temperature, Temp: temperature

ple size to show a statistically significant difference.

Temp	Time	Bicarbonate concentration (mmol/L)					
		13.5	95% CI	27	95% CI	54	95% CI
RT	Initial	10.2	[9.4, 11.0]	20.0	[18.7, 21.4]	42.1	[40.3, 43.9]
	2 h	9.5	[8.6, 10.4]	19.4	[17.7, 21.1]	40.8	[38.5, 43.1]
	24 h	9.4	[8.4, 10.5]	18.0	[15.8, 20.1]	39.8	[37.2, 42.4]
	<i>p</i>	0.44			0.26		
4°C	Initial	10.2	[9.4, 11.0]	20.0	[18.7, 21.4]	42.1	[40.3, 43.9]
	2 h	9.7	[8.8, 10.6]	19.4	[17.7, 21.1]	41.1	[39.0, 43.0]
	24 h	10.1	[8.8, 11.4]	19.0	[17.0, 21.0]	40.3	[37.8, 42.7]
	<i>p</i>	0.78			0.69		

Table S-4: DESCRIPTIVE AND INFERENTIAL DATA FOR THE EFFECT OF STORAGE TIME ON BICARBONATE RECOVERY STRATIFIED BY DECISION LIMIT – concentrations are in *mmol/L*, *p*: *p*-value of one-way ANOVA ($p \leq 0.05$ is significant), RT: room temperature

The descriptive data for TCO_2 as determined on the RapidLab[®] 348 blood gas analyser and the Beckman-Coulter DxC[®] analyser excludes normality using the Shapiro-Wilk test (Table S-5).

Given the absence of normality (Table S-5), the Wilcoxon test was used to test for significant differences between the ABG and DxC samples (Table S-6). The inferential data (Table S-6) shows statistically significant differ-

Decision level	Analyser	n	mean	median	5 th	95 th	p
13.5 mmol/L	DxC	47	9.9	9.3	6.5	16.3	0.01
	ABG	47	8.2	7.4	4	14.2	0.02
27 mmol/L	DxC	48	19.7	17.6	10.1	37.1	3.3×10^{-9}
	ABG	48	18.9	16.6	7.5	46	6.9×10^{-7}
54 mmol/L	DxC	48	41.1	39.3	30.3	50.9	2.9×10^{-6}
	ABG	48	39.0	37.1	24.8	53.7	2×10^{-3}

Table S-5: DESCRIPTION OF ABG AND DxC TCO_2 CONCENTRATION COMPARISON DATA – p : p -value of the Shapiro-Wilk test for absence of normality ($p \leq 0.05$ is significant), n : count, ABG: RapidLab[®] 348 blood gas analyser, DxC: Beckman-Coulter DxC[®] analyser

ences between the ABG and DxC results.

Decision level	n	p	difference	95% CI
13.5 mmol/L	47	2.2×10^{-7}	1.8 mmol/L	[1.3, 2.3]
27 mmol/L	48	6×10^{-3}	1.4 mmol/L	[0.45, 2.25]
54 mmol/L	48	5.8×10^{-5}	2.2 mmol/L	[1.28, 3.15]

Table S-6: SUMMARY OF WILCOXON-TEST COMPARISON BETWEEN ABG AND DxC BICARBONATE CONCENTRATIONS BY DECISION LEVEL p : – p -value of the Wilcoxon-test ($p \leq 0.05$ is significant), n : count, difference is the median differences between the ABG and DxC results and the 95% CI is the 95% confidence interval for the median of the differences

Although statistically significant differences have been detected between the ABG and DxC analysers, the contributions of these differences (S-6) are not significant when compared to either the imprecision of the measurement or the reference change values (Table C.1 and Table C.3 respectively).

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D. Supporting documentation

D1: An introduction to clinical acid-base theory

Of the original 5 properties of acids defined by Boyle[1], only 2 remain valid and are:

- change blue plant dyes to red and
- lose the above property on contact with bases.

Thus acids are recognised by these properties.

In clinical chemistry, traditional Henderson-Hasselbalch based theories[2] and a recent physicochemical theory[3] exist to explain acid-base disturbance. The evaluation of these theories requires that two basic science questions should be answered[4]. The first, a physical chemistry question, is upon which acid-base model is the theory based and the second, a physiology question, is does the theory explain an underlying mechanism/physiology/pathophysiology (ie the regulatory factors)? The final question is an applied science question (a clinical question): does one theory provide different/more management information than the other? To critique these theories one has to recognise that there are two realms in which the discourse occurs. The first realm or space is descriptive and is described in the language of mathematics, and the second space is explanatory and is in the language of physiology. The separation of these spaces (descriptive and explanatory) will be achieved with

the introduction of a distinct vocabulary for each.

D1.1 Nomenclature

The convention here is that the term ‘variable’ refers to unknowns in a mathematical equation and that variables can either be independent (determined experimentally) or dependent (calculated from independent variables) – terms that will also be reserved for mathematical descriptions. With regard to regulatable outcomes (like pH), the term ‘factor’ will refer to underlying pathophysiological mechanisms and can either be regulated (physiological processes actively manipulated to achieve a desired outcome) or unregulated (inevitable consequence of regulated factors to maintain physical laws) – where regulated and unregulated are reserved for the underlying pathophysiology. Factors can either be associated (a change in one factor cannot be achieved without changing the other) or unassociated (one factor can be changed arbitrarily without changing the the other). Similar terms in the various spaces are summarised in Table D1-1 where ‘variable’ and ‘factor’ are similar terms but only apply in the relevant space. Note that both regulated

Space Language	Descriptive Mathematical	Explanatory Physiological
	Variable Independent Dependent	Factor Regulated Unregulated associated unassociated

Table D1-1: SIMILAR TERMS IN THE DESCRIPTIVE AND EXPLANATORY SPACES

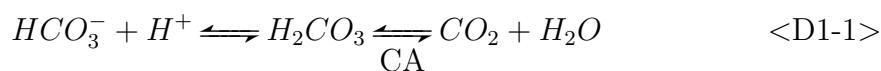
and unregulated factors can be either associated or unassociated and that there is no equivalent in the mathematical description of this dissertation.

D1.2 History of acid-base theory

Lavoisier proposed an oxygen-centric acid-base theory in 1776[1, 5]. This was followed by the hydrogen(proton)-centric[5] models of Arrhenius(1884) and then Brønstead-Lowry(1923). Since then, more comprehensive, electron-centric theories have been constructed by Lewis(1923 and restated in 1939)[1, 6], Usonovich(1939)[1, 5] and Pearson(1963)[7, 8] which explain the known acidic properties of non-hydrogen containing molecules and ions. In particular, Lewis acids are molecules, radicals or ions that can accept a non-bonding electron pair, in order to complete its valence shell, from another atom[1, 6, 9]. A brief introduction to Lewis-Acid base theory is provided in Appendix D3.

D1.3 Traditional acid-base theory in clinical chemistry

Bicarbonate (HCO_3^-), a base in the proton-centric theories[9], is considered fundamental to clinical acid-base regulation because, *in vivo*, it does not reach equilibrium as carbonic acid can dissociate into H_2O and CO_2 [2, 10] and the latter can be eliminated by the lungs. This dissociation is described by Reaction <D1-1>.



The dissociation of H_2CO_3 is catalysed by carbonic anhydrase(CA). In 1909 (six years after Arrhenius was awarded the Nobel prize in chemistry) the Henderson Equation (D1-1), a statement of the law of mass action for Reaction <D1-1>, was constructed:

$$[H^+] = K_1 \times \frac{[H_2CO_3]}{[HCO_3^-]} = K_1 \times \frac{[CO_2]}{[HCO_3^-]} \quad \text{(D1-1)}$$

where CO_2 is the dissolved carbon dioxide and HCO_3^- is the dissolved bicarbonate. Applying Sørensen's convention of expressing the H^+ concentration in pH units[11], such that

$$pH := -\log[H^+], \quad (D1-2)$$

to Equation (D1-1); the Henderson-Hasselbalch formula is derived:

$$pH = pK_1 + \log \frac{[HCO_3^-]}{[H_2CO_3]} = pK_1 + \log \frac{[HCO_3^-]}{[sCO_2 \times PCO_2]} \quad (D1-3)$$

where sCO_2 is the solubility of CO_2 and PCO_2 is the partial pressure of CO_2 . The right-hand-side of (D1-3) is the Henderson-Hasselbalch equation. It should be noted that (D1-1) was published before the Brønstead-Lowry acid-base theory and that the left-hand-side of (D1-1) is an Arrhenius acid. The latter statement is obvious when considering Reaction <D1-2>.



Nevertheless, when considering Reaction <D1-1>, HCO_3^- can also be considered a Brønstead-Lowry base (a superset for Arrhenius bases). Furthermore, the derivation is for an equilibrium state and is a one compartment model that only applies to the extra-cellular fluid. Finally, Henderson was aware of the buffering capacity of non-carbonate species[12, 13] but only considered them significant once the bicarbonate reserve had been significantly depleted[13] and would not have considered the effect of Lewis acids (serum cations like Na^+ and Ca^{2+} as discussed in Appendices D3.1 and D3.3). Given that this is a proton-centric model of acid-base disturbance, it cannot predict the effect of electrolytes on pH . Only PCO_2 and pH are measured directly[14]. There are a maximum of nine possible states (combinations of the independent variables)[15] and observation correlated these states with

combinations of altered respiration and altered net acid secretion (NAE) – a function of H^+ and HCO_3^- [15].

One should therefore deduce that, although the right-hand-side of Reaction <D1-1> forms a Brønsted-Lowry acid-base conjugate pair, the Henderson-Hasselbalch equation was based on an Arrhenius acid-base model [12, 13]. Equation (D1-3) only applies to the extra-cellular space at equilibrium when all the non-carbonate buffers and the electrolytes do not deviate substantially from normal [16] – the effect of Lewis acids is constant. In Equation (D1-3), PCO_2 and pH are independent variables and HCO_3^- is dependent but experimental data suggest that PCO_2 , pH and HCO_3^- are regulated factors. Thus dependent variables do not imply unregulated factors – proof provided in Appendix D2.1. The proof of the converse is provided in Appendix D2.2. Note that only Na^+ , Mg^{2+} , Fe^{3+} and Al^{3+} and NH_3 should in principle have the potential to modify acidity [8, 17]. Thus the Henderson-Hasselbalch equation deviates quantitatively when electrolytes are different from normal but may not deviate qualitatively. Finally it should be recognised that initially the Henderson-Hasselbalch equation was a description (not an explanation) and that only subsequent observation [15] and experiment could attach physiological explanation.

With the introduction of the van Slyke equation and base excess (BE) [14, 15, 18], effectively a two compartment model (incorporating intracellular haemoglobin) was applied to the principles underlying the original Henderson-Hasselbalch equation. Equation (D1-4) is a version of the van Slyke equation [19]

$$BE = [HCO_3^-] - 24.4 + (2.33[Hb] + 7.7) \times (pH - 7.4) \times (1 - 0.23[Hb]) \quad (D1-4)$$

where pH and HCO_3^- are calculated from (D1-3)[4, 18, 19].

Apart from the theoretical limitations above, practical limitations exist. PCO_2 and pH are independent variables in (D1-3) and are measured as such. However these factors are associated – at least one argument in the great trans-Atlantic debate[9, 20]. The Copenhagen group in the great debate circumvented the problem by calculating BE (the concentration of titratable H^+ required to return the pH of *in vitro* whole blood to 7.4 at 37°C, with PCO_2 held at 40mmHg[19]) from (D1-4) and introducing standard base excess (SBE) [21] – which standardises BE to a haemoglobin concentration of 5 g/dL[22]. However, this solution has also been criticised because the *in vitro* results do not not reflect the *in vivo* situation[10, 19, 21].

D1.4 Evaluating Stewart’s contribution

At least two components exist to Stewart’s contribution to acid-base physiology. The first is descriptive and the second is explanatory. With regard to description, given the discrepancy between *in vivo* and *in vitro* measurements, the option exists to circumvent the above problems associated with the direct measurement of H^+ by indirect measurement. The above action is only justified if no discrepancy exists between *in vivo* and *in vitro* measurement of the indirect factors. Stewart applied electroneutrality to determine $[H^+]$. Thus the difference between all the anions and cations should be a function of $[H^+]$ [3, 23]. Two differences should be recognised between Stewart’s approach and an earlier attempt (buffer base) in 1948[23]. The latter also considered the effect of non-carbonate buffers (grouping them into buffer base or SID) but did not isolate the non-carbonate buffers[23] for interpretation (the equivalent of $[A_{TOT}]$) and a flawed interpretation of acids and bases was used[24, 25].

D1.4.1 Acid-base model type

It is thus evident that Stewart's contribution is a proton-centric model and can therefore not explain the contribution of non-protonated ions to acidity. The proof that Stewart's theory is not compatible with more comprehensive theories in physical chemistry is provided in Appendix D2.3. Stewart's use of the water dissociation constant to determine the relationship between $[H^+]$ and $[OH^-]$ is consistent with an Arrhenius model of acid-base[3].

Thus, in theory, the model should provide no information beyond the constraints of an Arrhenius model. It has already been shown that the Henderson-Hasselbalch equation is also an Arrhenius theory for determining $[H^+]$. Thus, equating Stewart's formula[3] for $[H^+]$ to (D1-1),

$$[H^+]^3 = K_A K'_w + (K'_w + K_A ([A_{TOT}] - [SID])) [H^+] - (K_A + [SID]) [H^+]^2 \quad (D1-5)$$

$$= \left(\frac{sCO_2 PCO_2}{K_1 [HCO_3]} \right)^3 \quad (D1-6)$$

where either SID_{app} or SID_{eff} (which should be equal) can be substituted for SID . These are defined as[26, 27]

$$SID_{app} := [Na^+] + [K^+] + [Mg^{2+}] + [Ca^{2+}] - [Cl^-] \quad (D1-7)$$

$$SID_{eff} := [HCO_3^-] + [Alb^-] + [Pi^-]$$

$$[Alb^-] = [Alb] \times (0.123 \times pH - 0.631) \quad (D1-8)$$

$$[Pi^-] = [Pi] \times (0.309 \times pH - 0.469)$$

$$SID_{eff} \approx [HCO_3^-] + 0.28 [Alb] (g/L) + 1.8 [Pi] (mmol/L) \quad (D1-9)$$

$$A_{TOT} = 0.344 [Total\ protein] = 0.572 [Alb]. \quad (D1-10)$$

The Stewart theory adherents follow Equation (D1-5) and the traditional-

ists follow (D1-6). It has been shown explicitly that the one compartment descriptions are identical[16, 24, 28] such that (D1-3) can be restated as

$$pH = pK_1 + \log \frac{SID - [A_{TOT}]}{sCO_2 \times PCO_2}. \quad (D1-11)$$

It is counter-intuitive that a reduction in $[A_{TOT}]$, a buffer in the traditional approach, should produce alkalosis. However, in the Stewart approach, albumin is viewed as weak acid (potential proton-donor) thus a reduction in weak acid induces an alkalosis[26].

A two compartment model has been constructed by Wooten[29]. Figure D1-1 compares the two theories with regard to the available acid-base theories. Thus one can deduce that the original Stewart theory is based on

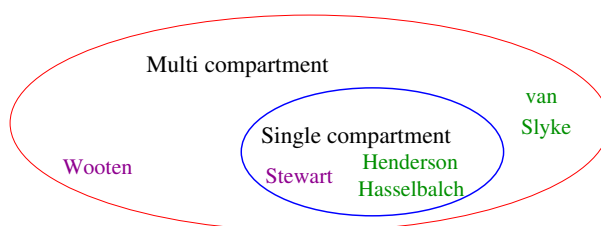


Figure D1-1: VENN DIAGRAM COMPARING THE NUMBER OF COMPARTMENTS DESCRIBED BY VARIOUS MODELS

an Arrhenius acid and is a one-compartment model that only applies in the extracellular space at equilibrium when the buffers and electrolytes do not deviate substantially from normal. The last statement is not obvious. The origin is that there are implicit assumptions about the dissociation of water in the presence of ions. In particular, the theory treats all ions as equal (which the Lewis acid-base theory refutes as demonstrated in Appendix D2.3). It is apparent that the original Stewart theory suffers from the same flaws as the Henderson-Hasselbalch theory and should also be inaccurate when electrolytes or proteins deviate substantially from normal[4]. The relationship between the clinical chemistry acid-base models and the physical chemistry

models is depicted in Figure D1-2.

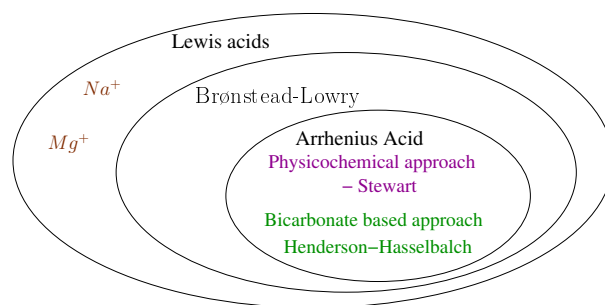


Figure D1-2: VENN DIAGRAM COMPARING THE CLINICAL CHEMISTRY MODELS TO THE PHYSICAL CHEMISTRY ACID-BASE THEORIES

At a practical level, in (D1-6), $[H^+]$ and PCO_2 are independent variables and $[HCO_3^-]$ is dependent whereas, in (D1-5), A_{TOT} and $[SID]$ are independent variables and $[H^+]$ is dependent. Although the difficulties associated with the measurement of *in vivo* factors *in vitro* are avoided using (D1-5) (because the results of the measurements are the same *in vitro* and *in vivo*), the uncertainty of measurement increases[30]. One has the option of using (D1-5) to calculate $[H^+]$ in (D1-6), measuring tCO_2 to calculate $CO_2 + HCO_3^-$ and then solving the simultaneous equations – which should provide compatible *in vitro* and *in vivo* results. In summary Stewart's theory is an alternate means of determining H^+ .

D1.4.2 Stewart theory physiology

The second component of Stewart's theory is the explanation of the physiology – the ion equilibrium model of acid-base demonstrates that this component is superfluous[10]. Similarly, it is unfortunate that the Henderson-Hasselbalch description is not separated from the physiological explanation[15]. Stewart deduces that independent variables imply and are implied by regulated factors and conversely that dependent variables imply and are implied by unregulated factors[3]. Intuitively one cannot deduce the underlying phys-

iology from the mathematical description[4] and the proof is provided in Appendices D2.1 and D2.2.

Although Stewart's deduction is flawed, the results may not be. The available evidence indicates that urine acidification is a combination of HCO_3^- reabsorption and H^+ secretion. The former is explained (using Stewart's approach) by HCO_3^- reabsorption, on the basolateral membrane of the proximal tubular cell (Figure B.2), being mediated by the kidney sodium bicarbonate co-transporter (kNBC)[31] – Na^+ increases SID [32] in Equation (D1-11). Similarly the secretion of H^+ , by the α -intercalated cells of the distal convoluted tubule requires the kidney anion exchanger (kAE)[31], on the basolateral membrane, to reabsorb HCO_3^- in exchange for serum Cl^- (Figure B.3) – increasing SID [32] in (D1-11).

Until the mechanism by which a putative H^+ or HCO_3^- sensor regulates kNBC and kAE directly or a putative SID or A_{TOT} sensor directly regulates kNBC and kAE is elucidated, it cannot be determined whether Henderson-Hasselbalch or Stewart respectively, explain which factors are regulated[4]. Note that H^+ is the strongest Lewis acid and if acidosis is regulated, it is appropriate that H^+ should be regulated directly. The alternative is to accept that acid-base disturbance is the penalty for electrolyte disturbance. Finally both descriptions accept the respiratory regulation of CO_2 which is directly related to H_2CO_3 and H^+ by (D1-3).

D1.4.3 Application of Stewart theory

Regarding diagnosis, in (D1-3) there are two independent and one dependent variable. Consequently there are nine possible states[15] of which seven (including normal) are recognised clinically[33]. Equation (D1-11) has three

independent variables and one dependent variable and consequently twenty-seven potential states exist. The number of potential states can, however, be increased arbitrarily (Appendix D2.4). Thus the number of potential states described is not a criterion by which to assess the clinical utility of each approach. Nevertheless, it is evident that Stewart's theory can identify more categories of acid-base disturbance – twelve categories (including normal) are recognised and summarised in Table D1-2[26]. Stewart's the-

	Acidosis	Alkalosis
Respiratory	$\uparrow CO_2$	$\downarrow CO_2$
Non-respiratory(metabolic)		
(a) Abnormal SID		
(i) Water excess/deficit	$\downarrow SID, \downarrow [Na^+]$	$\uparrow SID, \uparrow [Na^+]$
(ii) Imbalance of strong anions		
(α) Chloride excess/deficit	$\downarrow SID, \uparrow [Cl^-]$	$\uparrow SID, \downarrow [Cl^-]$
(β) Unidentified anion excess	$\downarrow SID, \uparrow [XA^-]$	
(b) Non-volatile weak acids (A_{TOT})		
(i) Serum albumin	$\uparrow [Alb]$	$\downarrow [Alb]$
(ii) Inorganic phosphate	$\uparrow [Pi]$	$\downarrow [Pi]$

Table D1-2: CLASSIFICATION OF PRIMARY ACID-BASE DISTURBANCES BY STEWART'S APPROACH[26]

ory is able to distinguish low SID acidosis being masked by compensating hypoalbuminaemia[33, 34]. Furthermore the acid-base disturbances listed in Table D1-2 may not traditionally be recognised as such but would be interpreted as hypo/hypernatraemia, hypoalbuminaemia or hypophosphataemia[24]. Usually the two approaches describe the same acid-base disturbance but the aetiology is interpreted differently[26, 33]. However, traditionally, for a finding of hypoalbuminaemia with metabolic acidosis, the hypoalbuminaemia would not be considered causal[24, 26].

Effectively, Stewart's approach (using SID) corrects HCO_3^- for partially dissociated ions ('weak acids'/non-carbonate buffers). The utility of correcting the $[HCO_3^-]$ for the contribution of albumin and phosphate has, traditionally, been considered insignificant[12, 13] and later not considered

because of its insignificance[23]. However, by 1928, there was evidence of significant albumin cation binding capacity[35, 36] – relevant when calculating the anion gap. *In vitro*, albumin buffering is significant[37–39] and plasma protein buffering capacity may be double that of bicarbonate[40]. *In vitro*, haemoglobin is responsible for 79% of the buffering and plasma (bicarbonate, protein and inorganic phosphate) is responsible for the remaining 21%[40]. *In vivo*, a rat model which determines whole blood buffering capacity in the presence of acute respiratory acidosis and alkalosis[41] suggests that albumin contributes 23% to the total buffering capacity[41] – despite the Sprague-Dawley rat having equivalent haemoglobin[42] and bicarbonate concentrations[43] and lower albumin[41] when compared to humans.

Ultimately, the consideration of phosphate and albumin as buffers depends on whether clinical management is altered. Figure D1-3 summarises the acid-base approaches by scenarios in which each test should apply best.

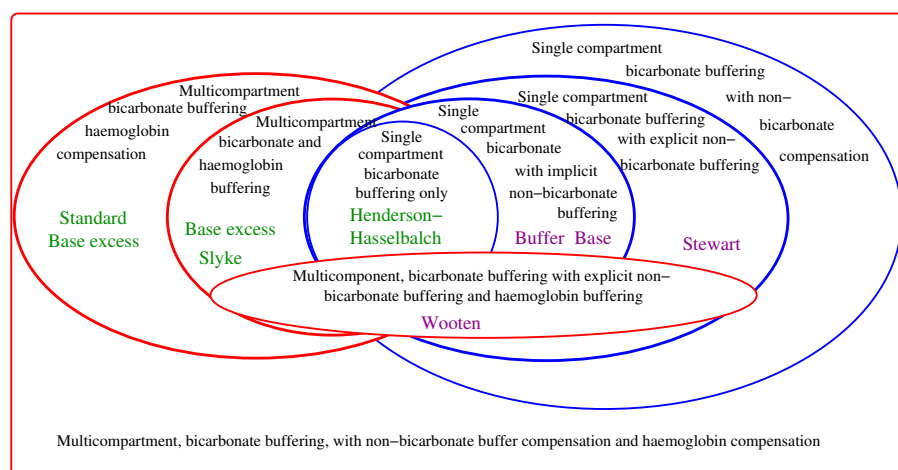


Figure D1-3: VENN DIAGRAM OF CLINICAL SCENARIOS TO WHICH THE VARIOUS ACID-BASE APPROACHES SHOULD APPLY

A benefit in the management of intensive care unit patients has not been shown[33, 44] despite Stewart’s theory being able to detect hypoal-

buminaemia masking an acidosis which is not recognised by base excess[33]. The equivalent of the anion gap in the traditional approach is the strong ion gap (SIG) [33] which is defined by the difference between (D1-7) and (D1-9)[26],

$$SIG = SID_{app} - SID_{eff}. \quad (D1-12)$$

SIG corrects for albumin and is more sensitive at detecting unmeasured anions[33, 45]. Note that albumin's contribution to SIG is $0.28 [Alb] (g/l)$ from equations (D1-9) and (D1-8). No benefit has been demonstrated in haemodiafiltration[46].

D1.5 Closing comments

The phenomenon of metabolic acidosis due to the infusion of sodium chloride cannot be explained by either the Henderson-Hasselbalch or Stewart approaches. It has been suggested that in normal (0.9%) saline $[Na^+]$ and $[Cl^-]$ are equal whereas in plasma $[Cl^-]$ is less, thus the net effect of the infusion is a reduction in SID and thus acidosis[34, 45]. From the Henderson-Hasselbalch perspective, *in vitro* all the variables in (D1-3) are diluted and consequently pH should be elevated (the difference between the $[H^+]$ and $[OH^-]$) and *in vivo* the reduction in $[H^+]$ is compensated by intracellular $[H^+]$ displacement and respiration. Similarly, from the Stewart perspective, *in vitro* $[H^+]$, SID and A_{TOT} are all reduced which is measured as an elevation in pH while *in vivo* the displacement of intracellular $[H^+]$ and respiratory compensation should result in alkalosis. These findings are obvious given that, depending on the approach, $SID - A_{TOT}$ is a proxy measure of HCO_3^- or *vice versa*. Sodium chloride in solution has a pH of 6.7 – 7.3 which is lower than whole blood pH .

The fundamental difference between the Henderson-Hasselbalch and Stewart approaches is that the latter considers the effect of partially dissociated ions (non-carbonate buffers/'weak acids'). The effect of albumin is not considered significant but this has not been shown explicitly. Although the *in vitro* buffer capacity experiment[40] and the rat model[41] suggest a greater role for non-bicarbonate buffering, the implicit constraints of the rat experimental design still leaves a space to question the *in vivo* buffering capacity of bicarbonate and the relative contribution of the non-bicarbonate buffers.

The absence of clear evidence of clinical benefit of the physicochemical over the bicarbonate approach can potentially be explained as either the contribution of plasma proteins is insignificant or, given that haemoglobin contributes 79% of the total buffering, both the physicochemical approach and the bicarbonate approach provide circumstantial/qualitative evidence of the acid-base status.

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D2: Dissertation proofs

D2.1 Proof that independent variables do not imply and are not implied by regulated factors

Here proof by contradiction is used. Two possible scenarios are explored. Assume that dependent variables *imply and are implied by* regulated factors. Assume that plasma CO_2 is unregulated. PCO_2 can be measured directly with a CO_2 electrode. Thus an example has been provided of an unregulated factor being associated with an independent variable.

Conversely, assume that plasma CO_2 is regulated. PCO_2 can be measured directly with a CO_2 electrode. Thus an example has been provided of a regulated factor being associated with an independent variable.

Thus examples have been provided where an independent variable can be associated with both regulated and unregulated factors. Thus independent variables do not *imply and are not implied by* regulated factors.

D2.2 Proof that dependent variables do not imply and are not implied by unregulated factors

Two possible cases are explored and proof by contradiction is employed. Assume that independent variables *imply and are implied by* regulated factors. Assume that plasma CO_2 is regulated. Ignoring the contribution of quantitatively smaller carbonate species, the total CO_2 can be defined as

$$tCO_2 = HCO_3^- + CO_2. \quad (D2-1)$$

Given Reaction <D1-1>, in an acidic environment all the HCO_3^- is reduced to CO_2 – allowing tCO_2 to be measured by a CO_2 electrode. Thus (D1-3) and (D2-1) form a system of equations in two unknowns (dependent variables – HCO_3^- , CO_2) and pH and tCO_2 are independent variables. Thus an example has been provided where a dependent variable (CO_2) implies a regulated factor (CO_2).

Conversely, assume that plasma CO_2 is unregulated. Again (D1-3) and (D2-1) form a system of equations in two unknowns (dependent variables – HCO_3^- , CO_2) and pH and tCO_2 are independent variables. Thus an example has been provided where a dependent variable (CO_2) implies an unregulated factor (CO_2).

Thus examples have been provided where a dependent variable can be associated with both regulated and unregulated factors. Thus dependent variables do not imply and are not implied by unregulated factors.

D2.3 Proof that Stewart's theory is not a special case of Lewis acid-base theory

Stewart's theory calculates $[H^+]$ as a function of the difference between anions and cations[1]. Thus equimolar amounts of aqueous $CaCl_2$ or $NiCl_2$ should produce the same pH . However, aqueous $CaCl_2$ is alkaline[2] and aqueous $NiCl_2$ is acidic[3] – compounds that Lewis' theory was designed to address.

D2.4 Proof that the number of potential states can be increased arbitrarily

By definition

$$SID = [Na^+] + [K^+] + [Ca^{2+}] + [Mg^{2+}] - [Cl^-]. \quad (D2-2)$$

Let, without loss of generality,

$$SID_{-Na} = SID - [Na^+]. \quad (D2-3)$$

Thus, substituting (D2-3) into (D1-11),

$$pH = pK_1 + \log \frac{[Na^+] + SID_{-Na} - [A_{TOT}^-]}{sCO_2 \times PCO_2}. \quad (D2-4)$$

Equation (D2-4) has four independent variables and one dependent variable and consequently has eighty-one potential states. Thus separating the individual components in SID and $[A_{TOT}^-]$ one can increase the number of states exponentially.

References

1. Stewart PA (1983) Modern quantitative acid-base chemistry. *Can J Physiol Pharmacol* 61(12):1444–1461
2. MSDS (2009), Materials safety data sheet: calcium chloride. URL <http://www.jtbaker.com/msds/englishhtml/c0357.htm>
3. MSDS (2008), Materials safety data sheet: nickel chloride. URL <http://www.jtbaker.com/msds/englishhtml/N3000.htm>

D3: Lewis acid-base theory

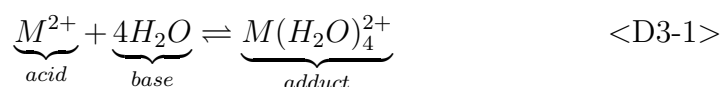
D3.1 Introduction

Before introducing the Lewis acid-base theory, one has to remove pre-conceived acid base theories. Firstly, one should not think of acids and bases in terms of H^+ . At the most basic level, acids change blue plant dyes to red and bases negate (or neutralise) this effect[1]. This phenomenon will be called the acid-base phenomenon and if the phenomenon occurs, the acid-base phenomenon criteria are fulfilled.

Arrhenius and Brønsted-Lowry identified a subset of compounds that could fulfill the acid-base phenomenon criteria. The subset of compounds identified by these authors all contained H^+ and consequently acid-base theories were constructed based on this subset of molecules and H^+ . The definition of acids as proton (H^+) donors are a construct of these theories and apply to this particular subset of molecules. The definition and theory are inseparable and the definition only applies to hydrogen containing molecules that satisfy the acid-base phenomenon criteria.

It must be recognised that the molecules identified by the protoncentric theories collectively form a subset of the superset of molecules satisfying the acid-base phenomenon criteria. In particular, non-hydrogen containing molecules can be shown to satisfy the acid-base phenomenon. Thus, by

observing molecules that satisfy the acid-base phenomenon, a larger subset of molecules are identified by the Lewis acid-base theory. The protoncentric molecules form a subset of the Lewis acids and bases. The theory constructed by Lewis defines an acid as a species that can form a covalent bond[2] by accepting an electron pair from another species[3]. A Lewis acid reacts with a Lewis base to form an adduct. All metal cations act as a Lewis acid when dissolved in water[2, 3] with the typical reaction



where M^{2+} is the metal cation and need not be of charge 2+.

D3.2 Proof that H^+ is a Lewis acid

When considering Reaction <D3-1>, it is evident that H^+ could be considered to be a metal cation. In such a case, it is evident that Arrhenius acids are contained within Lewis acids



where H_3O^+ is Arrhenius' hydronium ion.

Alternatively, hydrochloric acid is formed from the electron pair donor Cl^- which is a Brønsted-Lowry base and the electron pair acceptor H^+ – a Brønsted-Lowry acid – as described by Reaction <D3-3>



where HCl is covalently bonded.

D3.3 Proof that Lewis acids are acidic

The title can be rephrased as: “proof that Lewis acids cause an increase in $[H^+]$ ” and hence a paradox. The requirement for an increase in $[H^+]$ is a requirement of theory which is contained within the Lewis acid-base theory and only applies to a subset of Lewis acids and bases.

It cannot generally be shown that Lewis acids cause an increase in $[H^+]$ – and it is not necessary. Acids do not need to increase $[H^+]$, they only need to satisfy the acid-base phenomenon criteria.

Given that Lewis acids are a superset of the protoncentric models, it can always be shown that Arrhenius and Brønsted Lowry acids are examples of Lewis acids – and are thus acidic by the broader definition of Lewis acids.

References

1. Luder WF (1948) Contemporary acid-base theory. *J Chem Educ* 25:555–558
2. Ebbing D (1996) *General Chemistry*, chap. 15. Houghton-Mifflin, 641–642
3. Silberberg MS (2003) *Chemistry*, chap. 18. McGraw-Hill, 791–793

D4: Urine bicarbonate decision limits

D4.1 Calculation of urine bicarbonate decision limit

The fractional excretion of bicarbonate ($FeHCO_3^-$) is given by

$$FeHCO_3^- = \frac{U_{HCO_3^-}}{S_{HCO_3^-}} \times \frac{S_{Cr}}{U_{Cr}} \quad (D4-1)$$

where $U_{Cr} \approx 2.4 \text{ mmol/L}$ was arbitrarily selected as the urine creatinine concentration, $S_{Cr} \approx 0.1 \text{ mmol/L}$ is the serum creatinine concentration and $S_{HCO_3^-} \geq 22 \text{ mmol/L}$ is the minimum serum bicarbonate concentration required to calculate the $FeHCO_3^-$ [1, 2]. The decision limit is defined by a $FeHCO_3^-$ of 5% – below which one diagnoses RTA I.

Re-arranging (D4-1), a realistic urine bicarbonate decision limit is

$$U_{HCO_3^-} = FeHCO_3^- \times S_{HCO_3^-} \times \frac{U_{Cr}}{S_{Cr}} = 0.05 \times 22 \times \frac{2.45}{0.1} = 27 \text{ mmol/L}. \quad (D4-2)$$

Thus the urine bicarbonate decision limit will be 27 mmol/L and half the decision limit will be 13.5 mmol/L .

D4.2 Calculation of double urine bicarbonate decision limit

Double the decision limit is defined by a FeHCO_3^- of 10% – above which one diagnoses RTA II[1, 2]. Substituting $\text{FeHCO}_3^- = 0.1$ into (D4-2) one calculates that

$$U_{\text{HCO}_3^-} = 0.1 \times 22 \times \frac{2.45}{0.1} = 54 \text{ mmol/L} \quad (\text{D4-3})$$

is double the urine bicarbonate decision limit.

References

1. Rodríguez-Soriano J (2002) Renal tubular acidosis: the clinical entity. J Am Soc Nephrol 13(8):2160–2170
2. Bagga A, Bajpai A, Menon S (2005) Approach to renal tubular disorders. Indian J Pediatr 72(9):771–776

D5: Funding

NATIONAL HEALTH LABORATORY SERVICE



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 E-mail: kerrin.begg@nhls.ac.za

06th May 2010

Dr. R. Benjamin
 Department of Clinical and Laboratory Science
 University of Cape Town

Dear, Dr. R. Benjamin

K-Project Number: KNC 116
Commences: 1 JUNE 2010
Expires: 1 JULY 2010
Amount: R14 945,90
Investigator: DR. R. BENJAMIN
Department: CLINICAL AND LABORATORY SCIENCE
Title: CONSTRUCTION OF AN EXTERNAL QUALITY ASSURANCE
 (EQA) PROGRAM FOR URINARY BICARBONATE

I wish to advise you that your application for K-Project funding for your research project entitled "Construction Of An External Quality Assurance (EQA) Program For Urinary Bicarbonate", has met all criteria and has been approved. The Total budget costs are R14 945.90

Kindly quote the K-Project Number KNC 116 when ordering reagents. Your K-Project will commence on the 1 June 2010 and terminate on the 1 July 2010, at which time you will be required to present a report to the Research Committee.

With best wishes,
 Yours sincerely,


 DR K BEGG

Acting Executive Manager: Quality Assurance, Academic Relations

<p>Acting Executive Manager Quality Assurance Academic Relations & Research</p> <p>2010 -05- 06</p> <p>SIGN:</p> <p>COMMENT:</p>
--

cc Dr. J. King; Dr. P. Berman

D6: Consent form

DEPARTMENT OF CHEMICAL PATHOLOGY
UNIVERSITY OF CAPE TOWN

CONSENT FORM

Investigators: Dr Peter Berman, Dr Judy King, Dr Ryan Benjamin

**DESIGN OF AN EXTERNAL QUALITY CONTROL PROGRAM FOR
URINARY BICARBONATE**

A disease called renal tubular acidosis (RTA) exists. In its mildest form RTA manifests with short underweight babies and in the extreme form with kidney stones and death. In South Africa 250,000 new babies are identified as being wasted and stunted each year. These 250,000 babies should be investigated for RTA. The purpose of this study is to determine whether the tests that we can provide to identify and classify RTA are capable of fulfilling their function and to design a quality assurance program to ensure that they remain fit for purpose.

The urine that you provide will anonymously be tested for total carbon dioxide (the gas we exhale every minute). Additional substances will be added to your urine and be retested. The substances are ammonium bicarbonate (powdered baking ammonia) and sodium bicarbonate (baking soda).

I,, agree to participate in the above study and hereby give permission that urine may be taken, analysed and/or stored.

I understand that I will undergo the following investigations:

- i. Urine will be taken and tested for total carbon dioxide
- ii. I further understand that a portion of these urine samples will be stored for:
 - a) possible reanalysis
 - b) analysis when the study is completed, if additional information has become available
 - c) further research purposes, subject to the approval of the University of Cape Town Research Ethics Committee and that any information from such research will remain confidential
 - d) I understand the results of these tests will not become available to me

I have been informed that:

- a) the investigators are under the obligation to respect medical confidentiality
- b) No DNA will be stored and no genetic/DNA tests will be done on these samples
- c) All samples will be anonymised prior to being sent to the laboratories.

I UNDERSTAND THAT THIS STUDY WILL BE OF NO BENEFIT TO ME AND ONCE MY URINE HAS BEEN TAKEN, THERE WILL BE NO WAY TO TRACE IT BACK TO ME.

ALL OF THE ABOVE HAVE BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS HAVE BEEN ADEQUATELY ANSWERED

Participant: Name Date:

Participant: Sign Date:

Informed consent obtained by: Name
Sign

Witness: Sign:

D7: Ethics approval



UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6626 • Facsimile [021] 406 6411
e-mail: shuretta.thomas@uct.ac.za

07 May 2010

REC REF: 211/2010

Dr R Benjamin
Clinical & Laboratory Science
Chemical Pathology

Dear Dr Benjamin

PROJECT TITLE: DESIGN OF AN EXTERNAL QUALITY ASSURANCE PROGRAM FOR URINARY BICARBONATE

Thank you for submitting your study to the Research Ethics Committee.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study.

Approval is granted until 15 May 2011.

Please note that a witness does not need to sign an informed consent sheet unless the potential participant is illiterate and cannot read the consent form.

Please submit an annual progress report if the research continues beyond the expiry date. Please submit a brief summary of findings if you complete the study within the approval period so that we can close our file.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

Lesley Henry

PP

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

The Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

D8: Author instructions

This document has been formatted for the journal: Accreditation and quality assurance.



Accreditation and Quality Assurance

Journal for Quality, Comparability and Reliability in Chemical Measurement

Editor-in-Chief: Paul De Bièvre; Ernst-Heiner Korte

ISSN: 0949-1775 (print version)

ISSN: 1432-0517 (electronic version)

Journal no. 769

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Instructions for Authors

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Reference citations in the text should be identified by numbers in square brackets. Some examples:

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3. This effect has been widely studied [1-3, 7].

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Nugen S, Baeumner HJ (2008) *Anal Bioanal Chem* 391:451-454
- Article by DOI
Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. *J Mol Med*.
Doi:10.1007/s001090000086
- Book
South J, Blass B (2001) *The future of modern genomics*. Blackwell, London
- Book chapter
Brown B, Aaron M (2001) In: Smith J (ed) *The rise of modern genomics*, 3rd edn. Wiley, New York
- Online document
Healthwise Knowledgebase (1998) *US Pharmacopeia*, Rockville. <http://www.healthwise.org>. Accessed 21 Sept 1998

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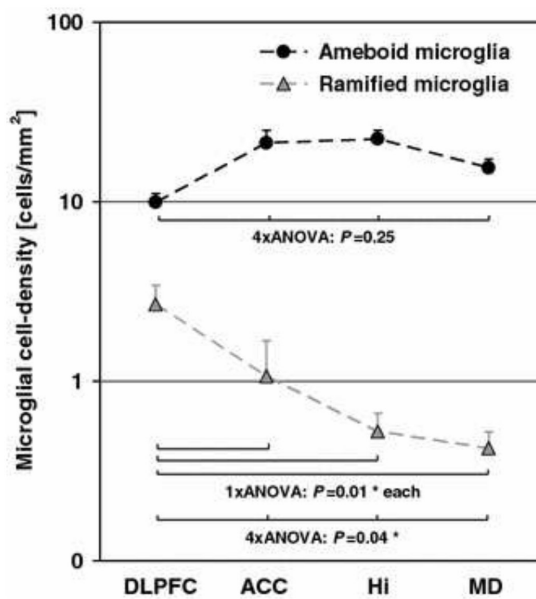
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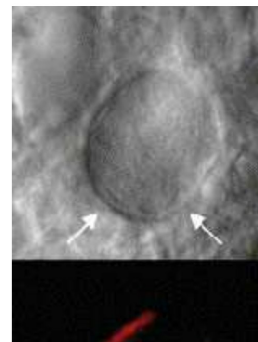
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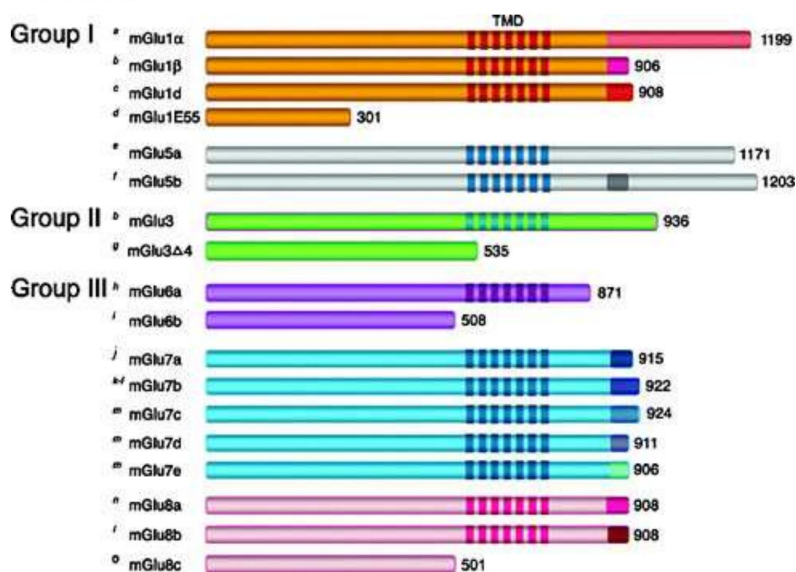
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D9: External quality assurance forms

D9-1 Proficiency testing instruction sheet

Attention:

Address of institution: HospLab

Dear Sir/madam

Thank you for participating in our urine bicarbonate external QA program. This is sample 09/2010/HospLab and consists of 6 specimens.

Enclosed you should find 6 microcentrifuge tubes containing various amounts of $NaHCO_3$. Two are 0.5 ml microcentrifuge tubes (can hold 550 μl). Two are 1.5 ml microcentrifuge tubes (can hold 1650 μl) and two are 2 ml microcentrifuge tubes (can hold 2200 μl). Each pair is labelled either 1 or 2. Below is a table listing the volume of urine to be added to the labelled microcentrifuge tubes (ml and μl). A total of 8 ml of urine will be required - note that volumes required differ among microcentrifuge tubes.

Any human urine may be used provided only one urine patient sample is used for all 6 specimens. Pooled urine can also be used. Tap the microcen-

Date	Tube size/tube name	Volume to add (ml)	Volume to add (μ l)	
30/09/2010	0.5 ml 1	0.5.1	0.475	475
	0.5 ml 2	0.5.2	0.475	475
	1.5 ml 1	1.5.1	1.425	1425
	1.5 ml 2	1.5.2	1.35	1350
	2.0 ml 1	2.0.1	2.2	2200
	2.0 ml 2	2.0.2	1.9	1900
Total	urine required ml	7.825		

trifuge tubes to dislodge the $NaHCO_3$ powder off the lid. Add the required volumes of urine to the microcentrifuge tubes, vortex and measure total CO_2 . Kindly return the results to me for analysis.

Your assistance is appreciated. I can be contacted at 021 404 4135 or ryan.benjamin@uct.ac.za or ryan.benjamin@nhls.ac.za.

Ryan Benjamin

Registrar: Chemical Pathology

National Health Laboratory Service,

Groote Schuur Hospital

and

University of Cape Town

D9-2 External quality assurance program report

QUALITY ASSURANCE PROGRAM REPORT URINE BICARBONATE

Name and site number of EQA program:	
Distribution number:	
Date sample received:	
Date sample processed:	
Date report back received:	

Report reviewed by:	Date:
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Comments

Approved by:	Date:
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Report submitted to QA manager	Date:
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B.Z. Toons

by Brian Zaikowski

www.bztoons.com



Sir, are you sure you sent me to the right planet?
'Cause I have my doubts about the people here being
ready for entrance into the intergalactic community.