Stability of 27 biochemistry analytes in storage at a range of temperatures after centrifugation

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ABSTRACT

Knowledge of factors affecting sample integrity is vital to make informed judgements on the validity of results. However, the information available for sample stability is incomplete, confusing and conflicting, particularly post-centrifugation. This study aims to investigate the effects of storage conditions on biochemical analytes. As part of this study, a new method has been developed, based on the manufacturer's stated analytical precision for the methodology. Ten adult volunteers were recruited into the study. Blood was collected into serum-separating tubes, and allowed to clot at room temperature for 30 minutes. After centrifugation, serum samples were stored frozen, refrigerated or at ambient temperature for between two hours and three months. After the allotted time had elapsed, designated serum aliquots were stored at –80°C, before batch analysis for 27 biochemical analytes. Twenty-three out of the 27 analytes remained stable until the last time-point tested at all temperature conditions. Alanine aminotransferase (ALT), lactate dehydrogenase (LD-P), potassium and uric acid showed reduced stability with at least one of the storage conditions tested. The method developed provided robust sample stability data within the inherent imprecision of the assay(s) used. The results generated can be used to create an evidence-based policy recommending sample handling and transportation practices that will ensure optimal sample integrity, and permit informed judgements to be made on results of stored samples. Minimal effects on sample stability were noted for the majority of analytes using the storage conditions tested in this study.

KEY WORDS: Biochemistry.
Reproducibility of results.
Time factors.

Introduction

Biochemical analysis of the chemical composition of body fluids is widely used to aid diagnosis and treatment. When serum/plasma is the specimen of choice, centrifugation of whole blood is a vital pre-analytical step to separate the cellular component from the fluid, thus minimising *in vitro* leakage of chemicals from red cells into the serum/plasma. The use of serum-separating tubes creates a physical gel barrier between the red cells and serum during centrifugation, largely eliminating the requirement to aliquot the serum.

In any laboratory, delays (planned or otherwise) may occur between sampling and analysis. As storage conditions of the sample, both pre-and post-centrifugation, are critical to maintaining the integrity of the samples, it is vital to be able to define exactly how delays in receipt and analysis may impact on the validity of results. However, although there appeared to be many sources of information pertinent to sample stability, including kit documentation, biochemistry reference textbooks, Clinical and Laboratory Standards Institute (CLSI) guidelines and published literature, the effects of storage conditions on sample stability are poorly investigated. The few studies[1–19] that have been published tend to focus more on the effects of delayed centrifugation, rather than on the effects of delays between centrifugation and analysis (Table 1). The focus of the present study is on post-centrifugation sample stability.

A literature search revealed incomplete, confusing and conflicting evidence for sample stability, due mainly to diverse definitions and methodologies (a conclusion also reached by other researchers).[3,7,13] A vital component, missing from many studies, is a robust definition of sample stability, which can then be used to guide the methodology used in its assessment.

This poor coverage of sample stability is not a recent phenomenon. Over 30 years ago, Thiers *et al.*[20] noted that many stability studies were superficial and lacked objective judgment. They proposed that stability should be assessed with reference to the analytical precision of the methodology used. Even though the paper provides an objective definition of sample stability, it has been largely ignored by subsequent researchers. Imeri *et al.*[21] recently recommended that the International Council for Standardization in Haematology (ICSH) should adopt a standard reference method to assess the stability of haematological parameters. However, no such recommendation has been made in relation to the stability of biochemical analytes.

The present study examines the hypothesis that storage conditions (time and temperature) have an effect on the stability of serum samples for biochemistry analysis post-centrifugation. The results of this study will be used to develop an evidence-based policy recommending best practices for sample handling and transportation to ensure optimal sample integrity. The storage conditions were chosen to meet current and projected future requirements of

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ICON Development Solutions (IDS) Clinical Pathology Laboratory, and included an assessment of the impact of the following issues (which are common to many laboratories):

- unforeseen delays in the laboratory leading to samples being left on the bench for up to one day prior to analysis
- samples delayed in transportation
- clients asking for repeat analysis or further tests many days after the original request
- effect of aliquoting the sample and storing the serum refrigerated, rather than storing the primary tube in the refrigerator
- remote sites choosing to freeze samples on site, and then sending them for batch analysis – is frozen storage at –20°C sufficiently low to preserve sample integrity, and what impact does multiple freeze/thaw cycles have on sample integrity?

After an extensive review of the literature, it became clear that knowledge of the analytical specification of the methodology in use, and, in particular, the manufacturer's precision guidelines, is vital for meaningful evaluation of stability data. However, only a few researchers[2,5,7,12,18] have

Table 1. Summary of literature reports for stability of serum/plasma.

Author(s)	Pre	Post	Temperature	Time	Number of samples	Tube type	Comments
Stokes *et al*.[1]		√	–15°C	0, 3, 6, 9, 12, 15, 18 weeks	30 pools	Serum	Cholesterol, HDLD, triglycerides only
Hill *et al*.[2]		√	4°C	0, 48 hours	88 pairs	BD plastic *vs.* glass	19 analytes (on the gel)
Heins *et al*.[3]	√	√	9°C, 25°C	1, 2, 3, 4, 7 days	20 fasting	Serum	22 analytes
Shih *et al*.[4]		√	–70°C	Up to 7 years	>150	Serum	Cholesterol, HDLD, triglycerides only
Woltersdorf *et al*.[5]		√	4°C	0, 2, 4, 7, 14 days	2 pools, 5 replicates	Serum	CK/AST/CKMB/TNT/ TNI, analysed as single batch
Boyanton and Blick[6]	√	√	25°C	0, 4, 8, 16, 24, 32, 40, 48, 56 hours	10 in duplicate	BD serum and plasma	24 analytes
Marjani[7]	√	√	4°C, 23°C	0, 1, 2, 3, 4, 5, 6, 7, 8, 24, 48, 72 hours	10 males	Serum	10 analytes only
Cramb *et al*.[8]		√	Freeze/ thaw cycles	1, 2, 3, 4 cycles	4–14	Serum	Lipid EQA
Jackson *et al*.[9]		√	4°C	0, 12, 24, 24, 36 hours	40	Serum	46 analytes
Jensen *et al*.[10]	√	√	10°C, 21°C, 30°C	4, 6, 8 hours	>400	Venosafe and BD plasma	16 analytes for transportation conditions
Ono *et al*.[11]	√		4°C, 23°C, 30°C	0, 2, 4, 6, 8, 24, 48 hours	10 females	Serum	25 analytes, batch-processed
Rehak and Chiang[12]	√		3°C, 10°C, 15°C, 22°C, 25°C, 30°C, 38°C	0, 24 hours	up to 20 samples	BD serum	29 analytes
Zhang *et al*.[13]	√		32°C	0, 3, 6, 24 hours	56 samples, but only 4–11 per analyte	BD serum	63 analytes
Clark *et al*.[14]	√		4°C, 21°C	0, 1, 2, 3, 4, 7 days	5 males, 7 females	BD plasma	13 parameters, batch-processed
Giltay *et al*.[15]	√		Room temperature	0, 1, 2 days	7 males, 13 females	BD serum	Cholesterol, HDLD, triglycerides, CRP, glucose, fatty acids
Foucher *et al*.[16]	√		4°C, 22°C	0, 1, 2, 4, 6, 8, 12, 24 hours	15	BD plasma	27 analytes
Tanner *et al*.[17]	√		15°C, 25°C, 35°C	0, 4, 8, 24 hours	15 males, 15 females	BD serum	35 analytes
Zwart *et al*.[18]	√		25°C	0, 1, 2.5, 5, 24 hours	11 fasting	BD serum	36 analytes, batch-processed
Leino and Koivula[19]	√		8°C, 22°C	0, 6 hours	50 fasting	Terumo plasma	26 analytes

Pre: Pre-centrifugation, Post: Post-centrifugation, BD: Becton Dickinson.

CRP: C-reactive protein, TNT/I: troponin T/I, EQA: External Quality Assessment scheme

See Table 2 for other abbreviations.

followed this guidance, and some use statistical analyses only. However, changes due to sample degradation and analytical variance within the limitations of the specified assay cannot be differentiated by statistical analysis alone. Stability studies normally take a single sample from each patient and analyse it at multiple time-points; a procedure which contains no physiological or biological variation. However, some researchers interpret sample stability using an element of biological variation,[17] which is inherently flawed. The only variable in a well-designed sample stability protocol should be analytical; a point also made by Jensen *et al.*[10]

After due consideration of all the methodologies used by the different research groups, it was decided to develop an in-house technique to evaluate the effects of storage conditions on sample stability post-centrifugation.[22] This technique was based on the principles outlined by Thiers *et al.*[20] and on the method developed by Livesey *et al.*[26] In contrast to the graphical truncated normal sequential test,[20] which requires a minimum of 15 samples and specialised statistical software, the in-house method used can provide robust results on 10 samples, using widely available computer programmes to analyse the data. The approach provides a graphical method of depicting sample stability, based on the manufacturer's stated analytical precision.

Analytes were defined as stable under specified storage conditions, when the differences between two or more measurements of this analyte were within the analytical precision defined by the manufacturer of the particular kits used (Beckman Coulter). The results obtained by this technique were compared to those obtained by statistical analysis of the same data, and to judgments found in the literature.

Materials and methods

Ethics approval

The study was carried out in partial fulfilment of the requirement for an MSc in Biomedical Science at the University of Ulster, and was approved both by the University of Ulster School of Biomedical Sciences Ethics Filter Committee, and the Manchester Independent Research Ethics Committee (MIREC). All volunteers gave signed, informed consent to participate in the study.

Volunteers

Ten volunteers (five male, five female) were recruited for the study. There were no restrictions placed on these volunteers, and all their details, other than gender, remained anonymous.

Study samples

A total of 54 mL of venous blood was collected from each volunteer into six Sarstedt Monovette serum-separating tubes (9 mL) via cannula, in accordance with IDS standard operating procedures. All samples were taken within a one-hour timeframe, and then allowed to clot for 30 minutes at room temperature, before being centrifuged at 1500 x*g* for 10 minutes.

Storage conditions

For each volunteer, the following procedure was adopted for the six tubes of blood:

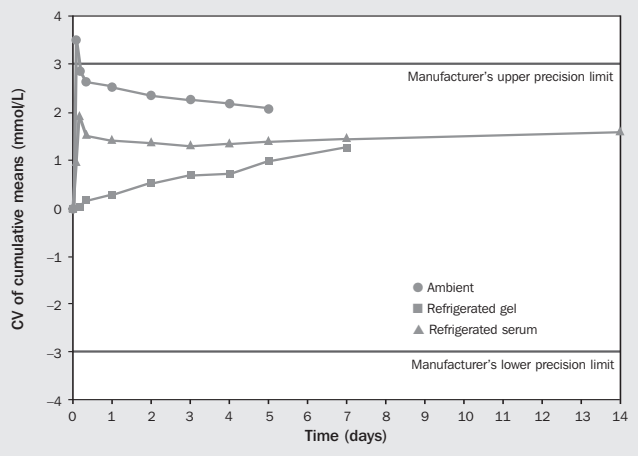

Fig. 1a. Cholesterol ambient and refrigerated stability assessment.

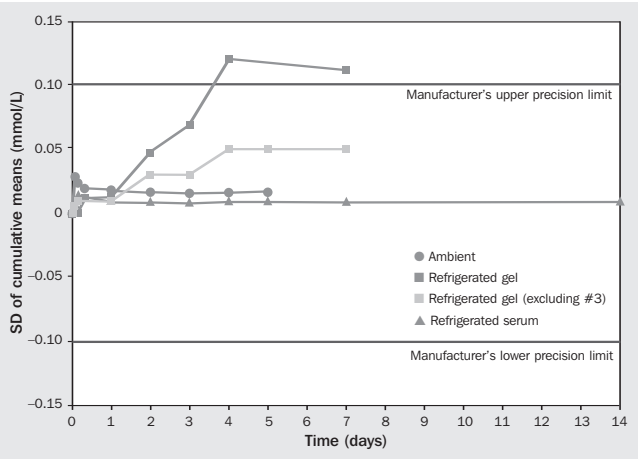

Fig. 1b. Potassium ambient and refrigerated stability assessment.

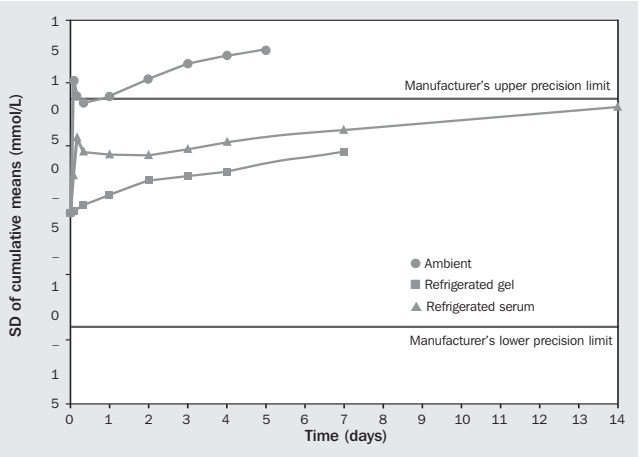

Fig. 1c. Uric acid ambient and refrigerated stability assessment.

- Two tubes were picked at random, and a serum aliquot from each analysed immediately to provide baseline values.
- A further tube (picked at random) was placed upright in the refrigerator at 2–10°C. Serum aliquots of 0.5 mL were taken at time-periods of 4, 8, 24, 48, 72, 96, 169 and 336 h, and stored at –80°C.
- All remaining serum was pooled and divided into multiple 0.5 mL aliquots, two of which were frozen immediately at –80°C to act as frozen baseline controls.

- Eight serum aliquots were stored at ambient temperature (18–25°C) for time-periods of 2, 4, 8, 24, 48, 72, 96 and 120 h, before being frozen at –80°C.
- Eight serum aliquots were stored refrigerated (2–10°C) for periods of 4, 8, 24, 48, 72, 96, 168 and 336 h, before being frozen at –80°C.
- Five serum aliquots were stored frozen at –20°C for periods of 1, 2, 4, 8 and 12 weeks, before being frozen at –80°C.
- Three additional serum aliquots were stored frozen at –20°C, and then subjected to one, two or three freeze/thaw cycles. For each freeze/thaw cycle, samples

Table 2. Analyte performance characteristics.

Analyte			BC Precision Guidelines			Analytical Range	Methodology	Chemistry Information Sheet	
Full Name	Abbreviation	Units	Changeover	SD	CV%			Reference	Date
Albumin	ALB	g/L	66.7	2.0	3.0	10–70	Timed endpoint	389705AD	Dec 07
Alkaline phosphatase	ALP	IU/L	85.7	3.0	3.5	5–1000	Kinetic rate	389706AD	Jan 08
Alanine aminotransferase	ALT	IU/L	85.7	3.0	3.5	5–400	Kinetic rate	389708AD	Jan 08
Amylase	AMY	U/L	85.7	3.0	3.5	5–800	Enzymatic rate	389712AE	Jan 08
Aspartate aminotransferase	AST	IU/L	85.7	3.0	3.5	5–400	Enzymatic rate	389714AD	Jan 08
Calcium	CALC	mmol/L	2.5	0.05	2.0	0.5–3.9	Ion-selective electrode	389722AE	Jan 08
Chloride	CL	mmol/L	100.0	2.0	3.0	50–200	Ion-selective electrode	389731AE	Dec 07
Cholesterol	CHOL	mmol/L	4.3	0.13	3.0	0.13–19.4	Timed endpoint	389726AD	Jan 08
Creatine kinase	CK	IU/L	142.9	5.0	3.5	5–1200	Enzymatic rate	389727AD	Jan 08
Creatine kinase MB isoenzyme	CKMB	IU/L	57.1	2.0	3.5	0.5–150	Modified Wurzberg assay	389729AD	Jan 08
Carbon dioxide	CO_2	mmol/L	33.3	1.0	3.0	5–40	Ion-selective electrode	389733AE	Jan 08
Creatinine	CR-S	µmol/L	600.0	18.0	3.0	27–2210	Modified rate (Jaffe)	A44572AB	May 08
Direct bilirubin	DBIL	µmol/L	52.0	2.6	5.0	1.7–171	Timed endpoint (diazo)	389739AD	Jan 08
Gamma glutamyl transferase	GGT	IU/L	85.7	3.0	3.5	5–750	Enzymatic rate	389745AD	Jan 08
Glucose	GLU	mmol/L	5.5	0.11	2.0	0.3–38.8	Timed endpoint	389746AD	Jan 08
HDL cholesterol	HDLD	mmol/L	2.3	0.07	3.0	0.13–3.5	Timed endpoint	389753AE	Jan 08
Lactate dehydrogenase	LD-P	IU/L	429.0	15.0	3.6	20–2500	Enzymatic rate	389768AD	Jan 08
Lipase	LIPA	IU/L	100.0	5.0	5.0	5–600	Enzymatic rate	389769AD	May 08
Magnesium	MG	mmol/L	1.6	0.03	2.0	0.04–2.88	Timed endpoint	389773AF	Jan 08
Phosphorus	PHOS	mmol/L	3.0	0.06	2.0	0.3–3.9	Timed endpoint	389785AF	Mar 08
Potassium	K	mmol/L	5.0	0.1	2.0	1–15	Ion-selective electrode	389761AF	Dec 07
Sodium	NA	mmol/L	100.0	1.0	1.0	100–200	Ion-selective electrode	389776AE	May 08
Total protein	TP	g/L	100.0	3.0	3.0	30–120	Timed endpoint (biuret)	389803AD	Jan 08
Total bilirubin	TBIL	µmol/L	86.7	2.6	3.0	1.7–513	Timed endpoint (diazo)	389795AD	Jan 08
Triglycerides GPO	TG	mmol/L	2.0	0.1	3.0	0.1–11.3	Timed endpoint	389796AD	Jan 08
Urea	UREA	mmol/L	24.0	0.7	3.0	1.8–35.7	Enzymatic rate	389808AE	Dec 07
Uric acid	URIC	µmol/L	450.0	9.0	2.0	30–714	Timed endpoint	389811AE	Jan 08

Taken from Beckman Coulter Chemistry Information Sheets[2]

were thawed at 37°C for five minutes, then mixed by inversion (x5) before being refrozen. There was a minimum period of 24 hours between cycles.

When the allotted incubation time was completed, the designated serum aliquot was stored at –80°C. This temperature is widely used for long-term storage,[4,5] and is the preferred storage temperature for UK Biobank[23,24] and other epidemiological studies.[14,25]

Subsequently, all the aliquots from each volunteer were divided into two batches for analysis: one batch contained all the samples originally stored at ambient temperature or refrigerated, and the other batch contained all the samples originally stored at –20°C, or subjected to freeze/thaw cycles. In addition, each batch included a frozen baseline sample. Batches were thawed at 37°C (to minimise cryoprecipitate formation), mixed by inversion (x5) and then analysed in a single analytical run. Although batch analysis entails the additional variable of a single freeze/thaw cycle, this approach is widely recommended as a method of reducing run-to-run variability.[5,11,14,18,26,27]

Analyses

All samples were analysed for 27 common biochemical analytes using a Beckman Coulter CX5 biochemistry analyser. This analyser is based on the Synchron platform, and shares the majority of reagents with the DX series of analysers. Table 2 summarises the performance characteristics of all assays performed.[28] Samples were assessed visually for sample integrity prior to analysis. Evidence of impaired integrity (e.g., haemolysis, serum-gel breakdown) was noted and used to inform the decision to exclude affected result(s) from statistical analysis.

Assessment of stability

The in-house technique used involved calculating the cumulative standard deviation (SD) and/or cumulative coefficient of variation (CV%), using the mean results of each time-point. This parameter was then plotted against the manufacturer's analytical precision limits (Table 2). An analyte was classed as unstable when the SD/CV% exceeded the stated precision limits of the analyser for that assay.[22]

Statistical analysis

Power calculations, using manufacturer's precision data and information obtained from the previous three months of internal QC data, indicated that an average of nine samples (range: 2–64 samples) would be needed to give an 80% chance of detecting sample instability at a significance level of 5%. Consideration of the cost and logistical implications of the study suggested that 10 subjects was the optimal number to guarantee provision of robust, good-quality scientific data. This number is not dissimilar to that used by other researchers.[5,7,8,11,13,14,26]

All data analyses were performed using a combination of Microsoft Excel 2007 and Analyse-IT version 2.20. Results were examined for normality by visual inspection of histograms, by coefficients of skewness and kurtosis, and by the Shapiro-Wilks test. The imprecision within-run and between-run for each temperature condition for parametric data was determined using analysis of variance (ANOVA). The corresponding imprecision for non-parametric data was determined using Kruskal-Wallis. Dunnett's post-hoc analysis was performed on any set of results where the ANOVA was significant.

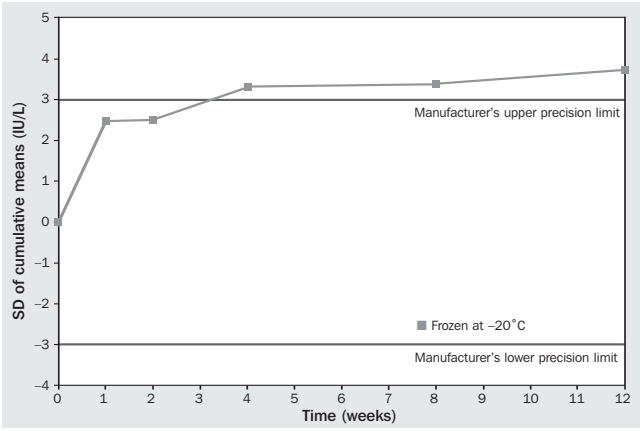

Fig. 2a. ALT frozen (–20°C) stability assessment.

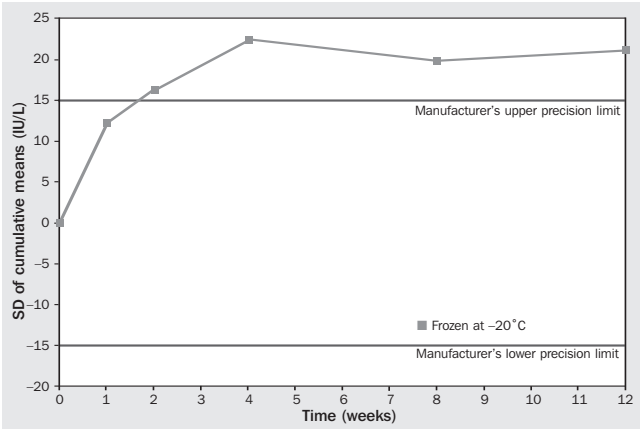

Fig. 2b. LD-P frozen (–20°C) stability assessment.

The majority of the primary gel tubes stored refrigerated contained insufficient volume to complete all the necessary analyses. As a consequence, limited results were obtained at 168 hours for each analyte (range: 2–9 results), although there was considered to be sufficient results to draw meaningful conclusions. At 336 hours, the only serum remaining (in limited volume) was from subjects #7 and #8: the results obtained from these two samples were significantly different from all other results. There was inadequate evidence to confirm whether this was due to sample instability or an error in sampling. Hence, there was insufficient data obtained at 336 hours for refrigerated storage on the gel, and no statistical analysis was performed at this time-point.

In addition, it was noted that the gel barrier from subject #3 started to break down from 96 hours onwards. This led to significant increases in results for potassium. Statistical analysis was performed with and without the potassium results for this sample at this temperature.

Results

Serum samples from 10 individuals were tested for 27 biochemical analytes at baseline and at six different temperature conditions over multiple different time-points. The mean (SD) for each analyte at baseline is given in Table

3, together with *P* values and stabilities for all storage conditions. The stabilities were calculated using the in-house technique, and indicate the time at which the manufacturer's recommended precision limits were exceeded. The calculated stability of selected analytes is shown graphically in Figure 1. A summary of the stability times achieved at refrigerated storage (in the primary tube on the gel, and as separated serum), together with comparative data from the literature, is given in Table 4. Each run contained internal quality control samples for the 27 analytes, as per the laboratory's protocol, and all results were within specified limits of acceptance.

Discussion

Literature review

Beckman Coulter recommends that serum samples for biochemical analysis should be stored at room temperature for no longer than 8 h, or refrigerated for up to 48 h,[28] with storage at –15 to –20°C being used for longer periods of storage. These recommendations match those given in the CLSI guidelines.[30] There are three exceptions to this advice, relating to the enzymes CK, CKMB and lactate dehydrogenase (LD-P).

There is limited sample stability information in biochemistry reference textbooks. In *Tietz Fundamentals of Clinical Chemistry*,[31] there is no mention of stability for 16 out of the 27 analytes tested in the current study, and very limited information for a further two analytes; the majority of the information relates to enzyme stability.[32] Furthermore, Beckman Coulter[28] and Tietz[31] are not in complete agreement for all analytes, in particular alkaline phosphatase (ALP) and alanine aminotransferase (ALT). This disagreement may be due to differences in methodology.

Both Beckman Coulter[28] and Panteghini[32] do not recommend frozen storage for LD-P, which fits with the findings of the current study that LD-P shows reduced stability on storage at –20°C and after freeze/thaw cycles. However, the current study found that, although some

Table 3. Summary of *P* values and stabilities.

				P values					Stabilities				
Analyte	Units	Baseline value Mean (SD)	Baseline	Ambient	Fridge gel	Fridge serum	Frozen –20°C	Freeze/ thaw	Ambient (hours)	Fridge gel (hours)	Fridge serum (hours)	Frozen –20°C (weeks)	Freeze/ thaw (cycles)
ALB	g/L	40.9 (1.1)	0.742	0.443	0.986	0.812	0.581	0.136	120	168	336	12	3
ALP	IU/L	62.4 (2.5)	0.950	1.000	1.000	1.000	0.996	0.983	120	168	336	12	3
ALT	IU/L	25.3v(1.2)	0.936	1.000	1.000	1.000	0.104	0.104	120	168	336	2	<1
AMY	U/L	58.1 (1.6)	0.999	1.000	1.000	1.000	1.000	1.000	120	168	336	12	3
AST	IU/L	23.8 (0.6)	0.979	0.868	1.000	0.980	0.911	0.663	120	168	336	12	3
CALC	mmol/L	2.26 (0.04)	0.180	0.996	0.964	1.000	1.000	0.971	120	168	336	12	3
CL	mmol/L	106.1 (0.8)	0.626	0.827	0.951	0.869	0.983	0.952	120	168	336	12	3
CHOL	mmol/L	4.56 (0.09)	0.970	0.999	1.000	1.000	0.999	0.976	120*	168	336	12	3
CK	IU/L	92.8 (2.3)	0.905	0.997	1.000	1.000	0.976	0.952	120	168	336	12	3
CKMB	IU/L	4.6 (0.8)	0.434	0.973	0.869	0.999	1.000	0.939	120	168	336	12	3
CO_2	mmol/L	27.4 (1.9)	0.008	0.982	0.891	1.000	0.941	0.778	120	168	336	12	3
CR-S	µmol/L	77.0 (5.6)	0.250	0.884	0.993	0.962	0.965	0.878	120	168	336	12	3
DBIL	µmol/L	1.5 (0.3)	0.524	0.185	0.967	1.000	0.985	0.834	120	168	336	12	3
GGT	IU/L	23.6 (1.1)	0.985	1.000	1.000	1.000	1.000	0.986	120	168	336	12	3
GLU	mmol/L	5.09 (0.08)	0.994	0.998	1.000	1.000	1.000	0.997	120	168	336	12	3
HDLD	mmol/L	1.30 (0.07)	0.729	0.886	0.992	1.000	1.000	0.997	120	168	336	12	3
LD-P	IU/L	348.5 (15.5)	0.744	1.000	0.963	0.346	0.283	0.094	120	168	168	1	<1
LIPA	IU/L	24.1 (1.6)	0.958	1.000	1.000	1.000	1.000	0.997	120	168	336	12	3
MG	mmol/L	0.89 (0.02)	0.948	0.834	0.986	0.979	0.986	0.986	120	168	336	12	3
PHOS	mmol/L	0.99 (0.04)	0.992	0.939	0.991	0.998	0.998	0.961	120	168	336	12	3
K	mmol/L	4.2 (0.06)	0.941	1.000	0.550	1.000	0.991	0.998	120	168†	336	12	3
NA	mmol/L	140.1 (0.6)	0.720	0.202	0.833	0.789	0.878	0.919	120	168	336	12	3
TP	g/L	67.8 (1.4)	0.403	0.515	0.999	0.947	0.991	0.789	120	168	336	12	3
TBIL	µmol/L	11.2 (0.9)	0.898	0.876	1.000	1.000	0.998	0.982	120	168	336	12	3
TG	mmol/L	1.06 (0.02)	0.999	0.984	0.999	1.000	0.997	0.928	120	168	336	12	3
UREA	mmol/L	5.2 (0.1)	0.993	1.000	0.998	1.000	1.000	0.991	120	168	336	12	3
URIC	µmol/L	359.5 (4.3)	0.994	0.650	0.997	0.950	0.928	0.668	<2	168	336	12	<1

*Cholesterol shows a 'spike' at 2 h ambient, but stable afterwards, †Potassium only stable if gel barrier intact.

See Table 2 for abbreviations.

deterioration is noted under refrigerated storage, LD-P is stable for 168 h (seven days) in the primary tube and as separated serum. This is not in full agreement with either Beckman Coulter or Panteghini.

Similarly, the current study is not in full agreement with either Beckman Coulter[28] or Panteghini[32] with regard to the stability of CK and CK-MB. Both sources claim that these two enzymes show poor stability. However, no loss of stability was demonstrated during any of the storage conditions tested for the duration of the study.

Alanine aminotransferase showed reduced stability when frozen at –20°C, or after one or more freeze/thaw cycles. Although Beckman Coulter[28] makes no mention of reduced stability under these conditions, Panteghini[32] comments that ALT activity is rapidly lost on storage at any temperature. Panteghini[32] also suggests that ALP may show increasing activity during refrigerated storage; the current study found no evidence to support this claim. However, neither Beckman Coulter[28] nor Tietz[31] provide information to back up the current study's findings that uric acid shows limited stability when stored at ambient temperatures or after undergoing one or more freeze/thaw cycles.

The available literature also does not include stability data for CO_2 stored frozen at –80°C. However, CO_2 analyses on serum are known to be problematical, and the value of any results thus obtained is debatable. The preferred methodology for assessing CO_2 concentrations is blood gas analysis.

There are known issues with the Beckman Coulter cholesterol method on frozen samples: lower total cholesterol results may be seen after freeze/thaw in samples with high triglyceride values due to a matrix effect.[8] This phenomenon was not observed in the current study, as all the triglyceride values were within the reference range. It is also unlikely to be encountered with routine laboratory samples, as the majority of samples are stored refrigerated rather than frozen.

The current study was designed to minimise variability due to differences in environmental conditions, reagent lot and calibration between analytical runs. Hence, samples were analysed in batches at the end of the study. This approach is recommended by a number of researchers.[5,11,14,18,26,27] However, batch analysis relies on the assumption that samples are stable when frozen and then thawed. In common with a number of studies, the temperature of choice for the current study was storage frozen below –70°C.[5,14,18] This had the added advantage that storage at –20°C could be included as one of the storage conditions investigated in the study.

Although few studies have considered storage below –70°C, this is the temperature of choice for Biobank[23,24] and epidemiological studies.[14,25] Claims of stability in excess of five years for storage below –70°C exist.[14] There is also literature available on storage of cardiac markers,[5] lipids[4] and some enzymes[32] at this temperature.

The instability of uric acid, at ambient temperatures and after one to three freeze/thaw cycles, in the current study was unexpected. However, the findings are in partial agreement with those of Heins *et al*.[3] who reported a small but continuous rise in uric acid at room temperature. Unlike the current study, Heins *et al*.[3] found that the rise did not exceed accuracy guidelines until 72 h. In contrast, both Marjani, and Boyanton and Blick[6] found that uric acid levels

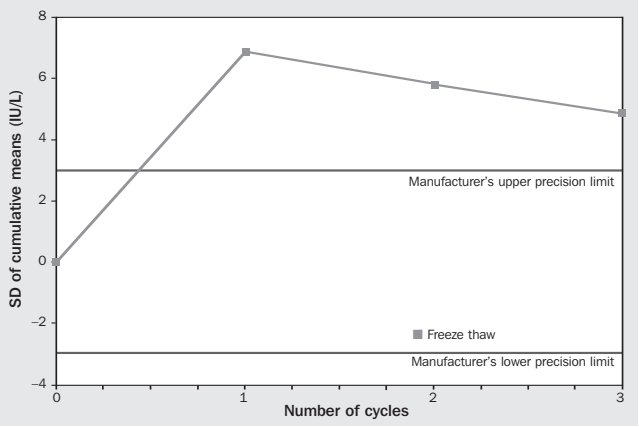

Fig. 3a. ALT freeze-thaw stability assessment.

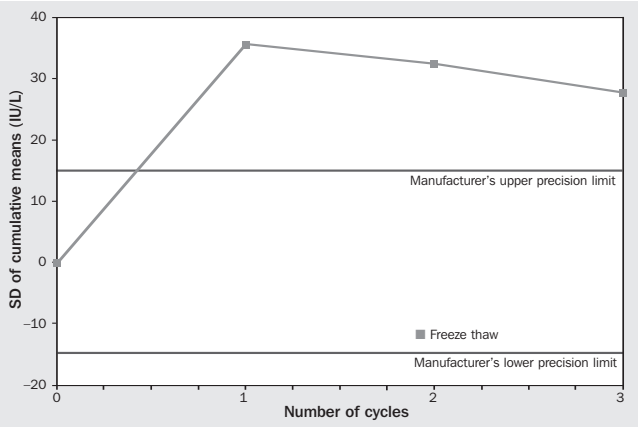

Fig. 3b. LD-P freeze-thaw stability assessment.

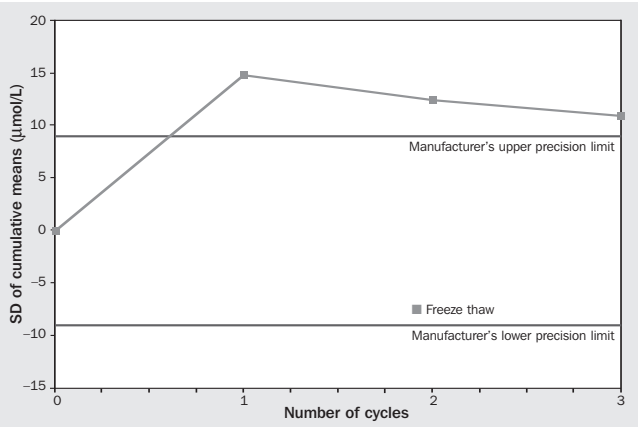

Fig. 3c. Uric acid freeze-thaw stability assessment.

showed a very slight decrease with time, although neither group regarded this as significant.

Seven other analytes (CK, creatinine, glucose, HDL, phosphate, total bilirubin, triglycerides) produced stability data at ambient temperatures in the current study that differ from those found by other researchers. In all cases, the statistical evidence supports the calculated stability, with the lowest *P* value being 0.876 (total bilirubin), and four *P* values exceeding 0.93 (CK, glucose, phosphate, triglycerides).

Boyanton and Blick[6] used a combination of statistical analyses and significant change limits (SCL) to determine that 24 analytes (including 23 of those included in the current study) were stable for 56 h at room temperature.

Marjani7 states that glucose, creatinine and phosphate showed reduced stability after only 24 h at ambient storage, whereas the other seven analytes tested were stable for 72 h. Although his stability assessment was made using statistics alone, the data provided in the paper indicate that the results for glucose, creatinine and phosphate exceeded the expected precision limits for those assays. Heins *et al*.[3] state that phosphate, total bilirubin, HDL, triglycerides and CK exceed accuracy guidelines (provided by the German Federal Council) after seven days' storage in the refrigerator.

The differences noted between this study and other research groups can be explained by the different methodologies used for analysis. There are five analytes (CO_2, LD-P, creatinine, glucose, phosphate) at refrigerated storage where the current study's findings are not in full agreement with the available literature. Hill *et al*.[2] found CO_2 to be unstable in the refrigerator. However, as discussed above, CO_2 measurements in serum are problematical. Although reduced stability after 168 h was demonstrated for LD-P, Heins *et al*.[3] report that LD-P is only stable for 96 h. Marjani[7] states that creatinine, glucose and phosphate are only stable for 48 h in the refrigerator. As these are the same three assays for which Marjani[7] demonstrates reduced stability at room temperature, this lack of stability could be due to differences in methodology. In addition, Jackson *et al*.[9] found a high probability of negative trends occurring with calcium, cholesterol and HDL within 36 h at refrigerated storage. They used random effects linear regression to ascertain stability, which they claim allows better compensation for variability of the assay and between individuals.

Sample stability assessment: analytical precision

The method developed in the IDS laboratory relies on the availability of precision information relevant to the methodology (kit plus analyser) being used for analysis.

Table 4. Comparative stability data for serum/plasma when stored refrigerated.

	Literature review					Study data	
	1992	1995	2001	2007	2008		
Analyte	Hill *et al*.[2]	Heins *et al*.[3]	Woltersdorf *et al*.[5]	Marjani[7]	Jackson *et al*.[9]	Gel	Separated
ALB	48 hours*	N/A	N/A	72 hours*	36 hours*	168 hours*	336 hours*
ALP	48 hours*	7 days*	N/A	N/A	36 hours*	168 hours*	336 hours*
ALT	N/A	7 days*	N/A	N/A	36 hours*	168 hours*	336 hours*
AMY	N/A	7 days*	N/A	N/A	36 hours*	168 hours*	336 hours*
AST	48 hours*	7 days*	7 days†	N/A	36 hours*	168 hours*	336 hours*
CALC	48 hours*	7 days*	N/A	72 hours*	36 hours*‡	168 hours*	336 hours*
CL	48 hours*	7 days*	N/A	N/A	36 hours*	168 hours*	336 hours*
CHOL	48 hours*	7 days*	N/A	72 hours*	36 hours*‡	168 hours*	336 hours*
CK	N/A	7 days*	14 days*	N/A	36 hours*	168 hours*	336 hours*
CKMB	N/A	N/A	7 days†	N/A	36 hours*	168 hours*	336 hours*
CO_2	Not Stable	N/A	N/A	N/A	36 hours*	168 hours*	336 hours*
CR-S	48 hours*	7 days*	N/A	48 hours†	36 hours*	168 hours*	336 hours*
DBIL	N/A	N/A	N/A	N/A	N/A	168 hours*	336 hours*
GGT	48 hours*	7 days*	N/A	N/A	36 hours*	168 hours*	336 hours*
GLU	48 hours*	N/A	N/A	48 hours†	36 hours*	168 hours*	336 hours*
HDLD	N/A	7 days*	N/A	N/A	36 hours*‡	168 hours*	336 hours*
LD-P	48 hours*	4 days†	N/A	N/A	N/A	168 hours*	168 hours†
LIPA	N/A	N/A	N/A	N/A	N/A	168 hours*	336 hours*
MG	48 hours*	7 days*	N/A	N/A	36 hours*	168 hours*	336 hours*
PHOS	48 hours*	4 days†	N/A	48 hours†	36 hours*	168 hours*	336 hours*
K	48 hours*	7 days*	N/A	N/A	36 hours*	168 hours§	336 hours*
NA	48 hours*	7 days*	N/A	N/A	36 hours*	168 hours*	336 hours*
TP	48 hours*	N/A	N/A	72 hours*	36 hours*	168 hours*	336 hours*
TBIL	N/A	7 days*	N/A	N/A	36 hours*	168 hours*	336 hours*
TG	N/A	7 days*	N/A	72 hours*	36 hours*	168 hours*	336 hours*
UREA	48 hours*	7 days*	N/A	72 hours*	36 hours*	168 hours*	336 hours*
URIC	48 hours*	7 days*	N/A	72 hours*	36 hours*	168 hours*	336 hours*

N/A: Information not available/analyte not tested in current study.

*Stable until end of study, †Reduced stability in current study.

‡High probability of negative trend, §Stable until end of study provided gel barrier intact.

See Table 2 for other abbreviations used.

Beckman Coulter provides comprehensive information sheets[28] for all its kits, which detail typical precision values that should be obtainable on a correctly maintained analyser, both for within-run precision and total precision assessments. This information includes a changeover value, an SD limit and a CV% limit. Results that are lower than the changeover value should be assessed against the SD limit, whereas those that are higher than the changeover value should be assessed against the CV% limit. The majority of results obtained in the current study (24/27 analytes tested) were below the changeover values, and hence stability was calculated against the stated SD limit. The exceptions were chloride, cholesterol and sodium: for these three analytes, the stability was calculated against the stated CV% limit. Cumulative SD/CV% was calculated at each time-point to exclude variability between subjects, so that an assessment of the variability between subjects could be made.

Using the method developed, all analytes were found to be stable under all storage conditions for the maximum time-period tested, with a few notable exceptions:

- ALT was only stable for two weeks frozen at –20°C, and not stable after freeze/thaw cycles
- cholesterol showed a 'spike' in the cumulative CV% value at 2 h at ambient temperature, but showed stability to 120 h (five days) afterwards – examination of the raw data revealed that cholesterol results showed an average 5% rise within the first 2 h, followed by a smaller steady increase with time; by 120 h, results were an average 6.3% higher than baseline values
- LD-P was only stable for one week frozen at –20°C, and not stable after freeze/thaw cycles
- potassium was stable in the primary tube stored in the refrigerator only if the gel barrier remained intact
- uric acid was not stable stored at ambient temperatures or after freeze/thaw cycles – examination of the raw data revealed that uric acid concentrations rise steadily on storage at ambient temperature, with results at 120 h an average of 11% higher than baseline values.

Sample stability assessment: statistical analysis

Statistical analysis of sample stability is widely used in the literature.[2,3,5–7,11,12,15,17–19] In the current study, ANOVA / Kruskal-Wallis tests were used to assess the suitability of the method developed in-house to generate robust stability data. ANOVA/Kruskal-Wallis tests make simultaneous comparisons between two or more means, and the resultant *P* value gives an indication of the significance of any differences between these means, where a *P* value <0.05 indicates that these differences are significant.

There was a wide spread of *P* values for each storage condition; however, the vast majority were greater than 0.95, indicating that the analytes were stable for that storage condition. The findings of the statistical analysis indicate that:

- 11 analytes were stable after freezing at –80°C – only one analyte (CO_2) showed statistically significant deterioration on freezing to this temperature, which was confirmed by post-hoc analysis using Dunnett's test
- 15 analytes were stable at ambient temperatures for 120 h
- 23 analytes were stable in the primary tube in the refrigerator for up to 168 h
- 22 analytes were stable as serum aliquots in the refrigerator for up to 336 h
- 20 analytes were stable frozen at –20°C for up to three months
- 15 analytes were stable after three freeze/thaw cycles.

Statistical methods were unable to confirm or refute stability of the remaining analytes, as *P* values were 0.05–0.95.

Sample stability assessment: comparison of methods

Overall, the current study found some correlation between the *P* values provided by ANOVA/Kruskal-Wallis tests and the stability data calculated from the manufacturer's specifications for precision. All analytes with $P>0.95$ were calculated to be stable until the last time-point tested for a given temperature condition, whereas the lowest *P* values tended to be associated with analytes showing the poorest calculated stability. However, there were a number of instances where results showed $P<0.9$ (and in some cases <0.5), and yet the analyte was stable until the last time-point tested (e.g., albumin and sodium at ambient temperature). Closer examination of the raw data for these analytes indicated a greater spread of results at each time-point, combined with increased variation for each subject between the different time-points, as shown by the raised CV%. However, it is not possible to use this increased variation in results as evidence that sample stability has deteriorated, as the changes were within the manufacturer's defined limits of the assay.

Conclusions

The current study showed that the original hypothesis that storage conditions impact on sample stability was only partially correct, as both statistical and calculated stability assessments demonstrated evidence of a deterioration in results on storage for some analytes. The in-house method used found that 23 of the 27 analytes were stable at the designated storage conditions for the time periods tested. Only four of the 27 analytes (ALT, LD-P, uric acid and CO_2) showed evidence of instability at one or more storage conditions.

As a result, the following conclusions can be made in relation to issues identified earlier:

- Serum samples for analysis of 26/27 analytes tested are stable for at least 8 h when stored on the laboratory bench at ambient temperature. The instability of uric acid was unexpected, and the findings are not backed up by the literature. This will now be discussed with the kit vendor, and further work may be needed to establish the limits of uric acid stability.
- Transportation delays of up to five days have no impact on the validity of results for 26/27 analytes tested in the current study (assuming temperatures during transportation are within the range 18–25°C). The exception was uric acid.
- Re-analysis or further analysis of serum samples for all 27 analytes can be made up to seven days after receipt of the sample without having any impact on the validity of the results, provided that the samples are stored refrigerated and the gel barrier remains intact.
- There are no advantages to aliquoting the sample and storing the serum refrigerated, rather than storing the primary tube. Equal stability was noted for all 27 analytes

using separated serum and serum stored in the primary tube in the refrigerator.

- Samples can be frozen at –20°C for up to three months, and subjected to three freeze/thaw cycles for 24/27 analytes with no loss of stability. Reduced stability for ALT and LD-P was noted both frozen at –20°C and after just one freeze/thaw cycle. Uric acid shows reduced stability after just one freeze/thaw cycle, but is stable for three months frozen at –20°C.

Statistical evidence correlated with this calculated stability in between 15 and 23 of the 27 analytes tested in the current study, depending on the storage conditions. The analytes where disagreement in stability was found between the two methods showed greater variation in results both within and between subjects at each time point tested. However, in most instances, this variation was found to be within the limitations of the assay.

Changes due to sample degradation, and analytical variance within the limitations of the specified assay, cannot be differentiated by statistical analysis alone. Hence, correlation with the statistical analyses and the available literature shows that the method developed in the IDS laboratory provides robust stability information about the inherent imprecision of the assay, and has confirmed that the current laboratory practice for the storage and analysis of biochemistry samples is fit for purpose.

The subjects used for the current study were typical of the populations used in clinical trials, and hence the results are of direct relevance to a medical laboratory setting. One limitation of the current study is that stability has not been assessed at pathological concentrations. However, all interpretations have been made based on the manufacturer's claimed precision limits for the assays. However, it is a possibility that analytes will demonstrate different stability profiles at higher concentrations than those tested here, due to non-linearity or hook effects. Thus, further work may be justified across a broader analytical range.

The results from the current study can be used to produce an evidence-based policy recommending best practices for sample handling and transportation to ensure optimal sample integrity. They can also be used to make informed judgements on results provided on stored samples. □

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