

Effects of storage duration and temperature conditions on biochemical analytes in porcine clotted, uncentrifuged blood samples

Abstract

Knowledge of factors influencing blood sample integrity is important to interpret results correctly. Effects of storage duration at different temperatures on porcine uncentrifuged blood samples are poorly described. The objective of this study was to elucidate effects of storage time and temperature on different porcine biochemical analytes. Multiple clotted, uncentrifuged blood samples from ten gilts were collected and samples were centrifuged 2h after sampling in the laboratory. Remaining samples were stored chilled (at $4\pm 2^{\circ}\text{C}$, CT) or at room temperature ($20\pm 2^{\circ}\text{C}$, RT). Samples were centrifuged 6, 12, 24, 48 or 72 hours after arriving at the laboratory. Storage characteristics and stability of albumin (Alb), calcium (Ca), creatinine (Creat), iron (Fe), gamma-glutamyltransferase (GGT), glutamate dehydrogenase (GLDH), glucose (Gluc), magnesium (Mg), inorganic phosphate (P), total protein (TP) and urea were studied. Test repeatability was within acceptable diagnostic limits. A significant time effect was found for Alb ($P<0.001$), Ca ($P<0.0001$), Creat ($P<0.0001$), GLDH ($P<0.0001$), Gluc ($P<0.0001$), Mg ($P<0.0001$), P ($P<0.0001$) and TP ($P<0.0001$). Over time, Alb, GLDH, Mg, P and TP increase while Ca, Creat and Gluc decrease. A temperature effect was identified for Ca ($P<0.05$), GGT ($P<0.05$), GLDH ($P<0.0001$), Gluc ($P<0.0001$), Mg ($P<0.0001$) and P ($P<0.0002$). All values, except Gluc, were higher when stored at RT. Gluc values were lower when stored at RT. This study highlights the importance of standardized storage routines of porcine clotted blood samples to ensure the validity of biochemical analyses.

Keywords: pig, biochemistry, storage, clotted, uncentrifuged, time, temperature

Volume 7 Issue 1 - 2018

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Received: November 27, 2017 | **Published:** January 08, 2018

Abbreviations: Alb, albumin; ANOVA, analysis of variance; BCS, body condition score; Ca, calcium; CV, coefficient of variation; Creat, creatinine; CT, cold temperature; Fe, Iron; GGT, Gamma Glutamyl transferase; GLDH, glutamate dehydrogenase; Gluc, glucose; H, hours; Mg, magnesium; P, inorganic phosphate; RT, room temperature; TP, total protein

Introduction

Correct interpretation of biochemical analytes is crucial for precise diagnosis and treatment of different diseases in production animals including pigs. Given the considerable distances between some farms and clinical laboratories in some countries like e.g. Norway, blood samples are sometimes delayed during shipment. If sample shipment should include a delay over weekend, transport duration may exceed 72 hours. During the summertime, temperatures in Norway can easily reach 25°C . On the other hand, winter temperatures in certain districts can occasionally drop below -30°C .¹ Previous studies confirm that storage conditions and transport duration of blood samples have a significant influence on biochemical values. Several studies have focused on storage duration and environmental effects on samples of human origin.²⁻⁷ Both normal serum activity and isoenzyme patterns differ between species and therefore it is not advisable to uncritically draw parallels between species.⁸ A few studies have looked at environmental and storage duration consequences on biochemical analytes in animal samples like sheep,^{8,9} cattle,^{10,11} horses¹² and dogs.^{13,14} Some studies have described effects on porcine blood fractions,^{8,15-17} a couple of these focused on the serum fraction.^{8,15}

To the authors' knowledge, only one study has been conducted on uncentrifuged clotted blood from pigs¹⁵ and this investigation looked at samples stored at room temperature. The aim of this study was to determine the analyte stability of porcine clotted, uncentrifuged blood samples stored at chilled temperature (CT) or at room temperature (RT) prior to centrifugation 2 hours to 72 hours post sampling. The wider objective was to be able to recommend standardized routines for sample storage and processing of blood samples from pigs.

Material and methods

Animals, sample collection and processing

Ten clinically healthy, five months old Norsv in Topigs gilts from a commercial sow pool center were used for this study. The gilts' body condition score (BCS) was evaluated and all gilts showed BCS between 3 and 3.5 (1 to 5 grading system). For blood sampling, the gilts were fixed with a rope around the upper jaw and blood samples were collected from the V. jugularis externa. Twelve tubes of 9 ml with micronized silica particles to activate efficient clotting (Vacuette® 9 ml, Med-Kjemi AS, Asker, Norway) were obtained from each animal. Tubes with separator gels were not used since gels can contain trace metals.¹⁸ The blood samples were transported in a closed, dark container to the laboratory, and arrival at the laboratory was within two hours after sampling. The samples from each animal were divided in two and stored either in a dark environment either at 4 or 20°C . The first set of samples were centrifuged ($3500\times g$ for 15 minutes (Megafuge 1.0 R, Heraeus SEPATECH, USA)) directly after arriving

at the laboratory (2h, RT). The remaining samples were centrifuge dafter storage durations of 6h, 12h, 24h, 48h and 72h, at CT or RT. To prevent thawing, freezing and thawing of the same sample, four serum aliquots of 2mL from each tube were frozen at -20°C in micro tubes (VWR, Hanover, Germany) until analyzed. In order to avoid concentration gradients during freezing and thawing,^{19,20} samples were mixed before analysis. The biochemical analyses were performed within two months after freezing.

Measurements of biochemical analytes

The following analytes were measured by applying the ABX Pentra 400 Analyzer (Horiba) for colorimetric analysis: albumin (Alb), creatinine (Creat), iron (Fe), glutamate dehydrogenase (GLDH), glucose (Gluc), gamma-glutamyl transferase (GGT), inorganic phosphate (P), total protein (TP), and urea. Calcium (Ca) and Magnesium (Mg) were measured by Atomic absorption (Analyst 300 Perkin Elmer). An overview of analytes, article numbers, methods and manufacturers is listed in Table 1.

Table 1 Methods for analysis of biochemical parameters

Analyte	Art. no	Product	Company	Instrument
Alb	A11A01664	ABX Pentra Albumin CP ³⁵	Bergman Diagnostic	ABX Pentra 400, Horiba
Ca	22296.294	Calcium Carbonate	VWR, Merck	A Analyst 300, Perkin Elmer
Creat	A11A01907	ABX Pentra Enzym. Creatinine CP ³⁶	Bergman Diagnostic	ABX Pentra 400, Horiba
Fe	A11A01637	ABX Pentra Iron CP ³⁷	Bergman Diagnostic	ABX Pentra 400, Horiba
GGT	A11A01630	ABX Pentra GGT CP ³⁸	Bergman Diagnostic	ABX Pentra 400, Horiba
GLDH	11 929 992 216	Glutamate dehydrogenase Cobas	Roche Norge	ABX Pentra 400, Horiba
Gluc	A11A01667	ABX Pentra Glucose HK CP ³⁹	Bergman Diagnostic	ABX Pentra 400, Horiba
Mg	25072.132	Mg ribbon ⁴⁰	VWR, Merck	A Analyst 300, Perkin Elmer
P	A11A01665	ABX Pentra Phosphorus CP ⁴¹	Bergman Diagnostic	ABX Pentra 400, Horiba
TP	A11A01669	ABX Pentra Total Protein CP ⁴²	Bergman Diagnostic	ABX Pentra 400, Horiba
Urea	A11A01641	ABX Pentra Urea CP ⁴³	Bergman Diagnostic	ABX Pentra 400, Horiba

The analytes measured by applying the ABX Pentra 400 Analyzer (Horiba) for colorimetric analysis were Alb, albumin; Creat, creatinine; Fe, iron; GLDH, glutamate dehydrogenase; Gluc, glucose; GGT, gamma-gamma-glutamyl transferase; P, inorganic phosphate; TP, total protein and urea; Ca, calcium; Mg, magnesium were measured by Atomic absorption (Analyst 300 Perkin Elmer).

Statistical analysis

Data analyses were performed using the commercially available statistical software Jmp (Jmp® Pro version 12.1.0, Cary, NC, USA). Normality and homogeneity of variance assumptions were graphically assessed by evaluating histograms and scatterplots, skewness and kurtosis, and by the Shapiro-Wilks test. Biochemical analytes in a reference sample were measured repeatedly (n=18) and the coefficient of variation (CV) was calculated to assess test repeatability. All results were within specified limits of acceptance. The sample stability under different storage conditions for parametric data was

evaluated by applying analysis of repeated measures (MANOVA, multivariate analysis of variance). The sample stability under different storage conditions for non-parametric data was determined using Kruskal-Wallis. Post-hoc testing by Tukey’s HSD test was used for multiple comparisons of the variables where the MANOVA was significant (P<0.05). Values of the biochemical constituents centrifuged twohours after sampling at RT were set to 100%. Values measured at 6h, 12h, 24h, 48h and 72h, at CT or RT after centrifugation were calculated as a percentage of the values achieved on samples 2h after centrifugation (Table 2).

Table 2 The contents of Alb, Ca, Creat, Fe, GGT, GLDH, Gluc, Mg, P,TP and Urea in blood samples from gilts according to storage and temperature conditions and percentage alterations (%)

Parameter	Unit	2 h RT (± SD)	Temperature	6h(%)	12h(%)	24h(%)	48h(%)	72h(%)
Alb	g/ L	37.8 (2.1)	CT	100	97	100	104	100
			RT	100	98	99	106*	104
Ca	mmol/ L	2.7 (0.1)	CT#	98	94*	90***	93*	99
			RT##	98	97	95	99	98
Creat	U/ L	120.7 (15.2)	CT	106	93	89	87	83**
			RT	107	93	90	90	88
Fe	µmol/ L	21.9 (4.1)	CT	101	98	87	98	96
			RT	101	102	89	101	103

Table Continued..

Parameter	Unit	2 h RT (± SD)	Temperature	6h(%)	12h(%)	24h(%)	48h(%)	72h(%)
GGT	U/ L	41.3 (6.8)	CT#	103	97	102	97	98
			RT##	105	106	108	113	112
GLDH	U/ L	2.03 (0.4)	CT#	59	57*	57*	84	141*
			RT##	79	57*	121	222***	391***
Gluc	mmol/ L	4.5 (0.8)	CT#	95	88	79	75*	71**
			RT##	86	67**	46***	31***	12***
Mg	mmol/ L	0.8 (0.1)	CT#	93	100	100	113	113
			RT##	100	113	150*	175**	213***
P	mmol/ L	2.7 (0.2)	CT#	100	98	111*	117***	121***
			RT##	104	109	148**	212***	326***
TP	g/ L	66.3 (3.3)	CT	103	89***	104*	107*	103
			RT	103	96*	106*	108*	110*
Urea	mmol/ L	2.9 (0.9)	CT	100	98	98	105	105
			RT	103	100	108	121	130

Percentage alterations of the parameters albumin (Alb), calcium (Ca), creatinine (Creat), iron (Fe), gamma-glutamyl transferase (GGT), glutamate dehydrogenase (GLDH), glucose (Gluc), magnesium (Mg), inorganic phosphate (P), total protein (TP) and urea in blood samples from gilts are shown. The values measured in the clotted blood samples 2 h after sampling (RT) are given as mean values±SD. Consecutive values are given as percentage of these values. A significant time effect was found for Alb, Ca, Creat, GLDH, Gluc, P, Mg, P, and TP

The superscripts *, ** and *** show significant differences from values measured at 2 h post sampling at significance levels of 0.05, 0.01 and 0.001, respectively

Different superscript signs (#, ##) show a significant temperature effect (P<0.05)

CT, cold temperature (4°C); RT, room temperature (20°C)

Results and discussion

The measured biochemical constituents after 6h, 12h, 24h, 48h and 72h, at CT and RT were evaluated relative to values at 2h (RT). Values at 2h (RT) were set to 100%. The numbers are discussed below and shown in Table 2. Repeated measurement analysis showed an overall significant time and temperature effect (P<0.0001). The time and temperature effects for each parameter are listed in Table 3.

Total protein (TP) and albumin (Alb)

A significant time effect was found for TP (P<0.0001) and Alb (P<0.001). TP serum mean concentrations in samples stored at CT or RT ranged from 89% to 110% of the mean concentration measured 2h post sampling while Alb mean concentrations ranged from 97% to 106%.

Calcium (Ca), iron (Fe), magnesium (Mg) and inorganic phosphate (P)

Significant time and temperature effects were found for Ca (P<0.0001). The values remained more stable when stored at RT compared to CT, ranging from 90% to 100% of the mean value measured at 2h post sampling. Significant time and temperature effects were found for Mg (P<0.0001). Mg concentrations changed significantly with increasing storage duration at CT and RT. However, less Mg changes were registered when samples were stored at CT. Storage of both 48 and 72 hat CT gave Mg concentrations of 113%. When stored at RT, a rise to 175% and 213% were found after 48h and 72h, respectively. Significant time and temperature effects were found

for P (P<0.0001). Serum samples stored at both CT and RT, showed a significant increase in P concentrations when stored over time. Again, less P concentration changes were registered when samples were stored at CT. No significant changes in Fe values were found.

Creatinine (Creat), glucose (Gluc), and urea

A significant time effect was found for Creat (P<0.0001). Samples stored at CT and RT for 72h showed Creat mean levels at 83% and 88% of the values measured 2 h post sampling, respectively. Significant time and temperature effects were found for porcine Gluc concentrations (P<0.0001). A storage period of 6h at CT led to mean Gluc concentrations that were 95% of the values measured at 2h. When stored at RT, the mean values were down to 86%. At CT and storage duration of 12h, the mean value was 88%. The corresponding mean value at RT was 67%. Finally, after 72h, Gluc concentrations of 71% and 12% were found at CT and RT, respectively. No significant changes in urea mean concentrations were found.

Gamma-glutamyl transferase (GGT) and glutamate dehydrogenase (GLDH)

A significant time and temperature effect was found for GLDH (P<0.0001). A rise in enzyme levels was found during the experimental period, both at CT and RT. However, enzyme levels reached higher levels when stored at RT. A temperature effect was found for GGT (P<0.05), and greater alterations in enzyme levels were found when samples were stored at RT. A total of 10 gilts were included in this study to provide robust scientific data. This number is not unlike that used by other researchers in both human and animal blood sample

stability studies.^{11,14,15,21–24} The study clearly shows that the stability of different relevant porcine blood analytes depend on both temperature levels and storage durations. Clotted blood samples were centrifuged 2 h after sampling or left at 4°C or 20°C for time intervals of 6h, 12h, 24h, 48h and 72h to simulate potential storage conditions and durations of porcine blood samples before reaching the laboratory. In general, few reports describe effects of temperature and storage duration on biochemical analytes in clotted blood of farm animal species,^{11,15–17,25} a couple of these include pigs.^{15–17} Dissimilarities in e.g. Gluc uptake and metabolism by erythrocytes exist across different species,^{11,12,26–30} demonstrating the importance of drawing up species-specific recommendations. Based on previous reports, clinically acceptable limits (CAL) in changes by storage for the analytes Mg, Creat, urea, Gluc, Fe, P and GGT can be set to 10%. With regards to Ca, Alb and TP, a CAL of 5% can be defined.^{3,15} To the authors' knowledge, only one published study describe the effects of storage duration and the anticoagulant additive heparine on analytes in uncentrifuged porcine blood samples, but this work was only done at room temperature (RT).¹⁵ The general trends in several biochemical analytes analyzed in the study of Framstad et al.¹⁵ and this study are similar. After a period of 48 h storage without cooling, GGT values obtained in this study or by Framstad et al.¹⁵ exceeded the defined CAL of 10%.

Table 3 Time and temperature effects on biochemical blood parameters

Components	Time	Temperature
Alb	<0.001	0.91
Ca	<0.0001	<0.0001
Creat	<0.0001	0.73
Fe	0.3	0.65
GGT	0.94	<0.05
GLDH	<0.0001	<0.0001
Gluc	<0.0001	<0.0001
Mg	<0.0001	<0.0001
P	<0.0001	<0.0001
TP	<0.0001	0.18
Urea	0.38	0.16

Time and temperature effects on the parameters albumin (Alb), calcium (Ca), creatinine (Creat), iron (Fe), gamma-glutamyl transferase (GGT), glutamate dehydrogenase (GLDH), glucose (Gluc), magnesium (Mg), inorganic phosphate (P) total protein (TP) and urea, in blood samples from gilts.

Significant time effects were identified for the parameters Mg, Ca, Creat, Gluc, P, Alb and TP. Temperature effects were found for Mg, Gluc, GLDH and P.

The sample stability under different storage conditions for parametric data was evaluated by analysis of repeated measures (MANOVA, multivariate analysis of variance).

The sample stability under different storage conditions for non-parametric data was determined using Kruskal-Wallis.

The increase of the mainly membrane bound GGT in serum samples stored at room temperature is due to oxidative damage of RBC during storage, both liberating cytoplasmic GGT and membrane bound GGT. Lower storage temperature will delay metabolic processes,³¹ slowing down cell membrane damaging processes. Storage at CT for 48h in this study led to a value decrease of 3%, within the defined CAL set for GGT. GLDH values were influenced both by time and

temperature conditions. Even though GLDH values did rise with more than 300% when stored at RT for 72h, the absolute GLDH levels were within the reference range.³² A strong Gluc decrease was found in samples stored non-cooled both in this study and by Framstad et al.¹⁵ This study gave a mean value of 46% after 24h storage at RT while their study gave a value of 67%, both far beyond the predetermined CAL of 10%. Although the mature erythrocyte of the pig has been observed to possess the slowest metabolic rate of any mammalian cell type, pig erythrocytes metabolize low but appreciable amounts of glucose to meet energy requirements,^{33,34} which explains why uncentrifuged samples will drop rapidly in Gluc concentrations post sampling. Again, as mentioned above, lower storage temperature will slow down metabolic processes. Gluc concentrations in samples stored at CT dropped slower than samples stored at RT. This study also shows that P and Mg increase more rapidly when stored at RT. This is in accordance with previous reports, highlighting the importance of slowing down metabolic processes by cooling down uncentrifuged, clotted blood samples from pigs prior to processing at the laboratory¹⁵ since erythrocytes will leak P into the serum post sampling.¹⁸ Differences in the degree of analyte increase or decrease between the study of Framstad et al.¹⁵ and this study may be due to the fact that temperature conditions may have been somewhat different and that the samples in this study were frozen before analysed. The authors acknowledge that freezing the samples prior to the analyses deviates from the routine practice of many laboratories. Additionally, tubes, laboratory equipment and reagents used for analyses have changed. Despite some differences in the degree of analyte increase or decrease, the general trends are very similar.

Conclusion

The results from this study show a significant time and temperature influence on analytical results in uncentrifuged, clotted blood samples from pigs. This study gives helpful guidelines for Veterinary practitioners and researchers in handling clotted blood samples from pigs. Laboratories can also benefit from the results, although samples were frozen after centrifugation. Serum tubes with separator gels can prevent metabolism effects by forming a barrier between the cellular and the serum fraction in the tube, which can be of advantage if the serum cannot be separated prior to shipment of the samples.

If no serum tubes with separator gels are available and the RT storage time before analysis is anticipated to exceed 24hours, the samples should indeed be centrifuged before shipment. If no centrifuge is available, the addition of cooling elements can slow down alterations. Some parameters like Gluc, Mg, P crossed the predetermined CAL already after 6h, 12h and 24h at RT, respectively, and such alterations may lead to misinterpretations of results. Finally, analytical results from uncentrifuged porcine blood samples should always be interpreted with caution according to the shipment duration.

Acknowledgements

The authors wish to acknowledge the farmer involved in this study for making sampling of his gilts possible. The sampling of the animals were conducted according to the current Norwegian Animal Welfare legislation Act and the EUs Directive 2010/63 on the use of animals for scientific purposes.

Conflict of interest

No financial interests or conflict of interests exist.

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