Clinica Chimica Acta 433 (2014) 278–283

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

The storage stability and concentration of acetoacetate differs between blood fractions

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ARTICLE INFO

Article history:
Received 6 December 2013
Received in revised form 27 March 2014
Accepted 27 March 2014
Available online 8 April 2014

Keywords:
Acetoacetate
Storage stability
Deproteinised
Blood fractions

ABSTRACT

Background: Plasma concentrations of 3-hydroxybutyrate (3HB) are measured more often than acetoacetate (AcAc) which may be due to the reported storage instability of AcAc. The aims of the study were to compare the storage stability of AcAc in different blood fractions over time (90 days) when stored at −80 °C and to determine the postprandial concentration of AcAc in whole blood, plasma and red blood cells.
Methods: Blood was collected from fasting subjects (n = 5): whole blood, plasma and red blood cells were isolated and deproteinised in perchloric acid, and supernatants were stored at −80 °C until analysis. Postprandial concentrations of AcAc in whole blood, plasma and red blood cells were determined at regular intervals over 420 min, after subjects (n = 23) had consumed a mixed test meal.
Results: Storing deproteinised plasma at −80 °C resulted in no significant change in AcAc concentration over 60 days. In contrast, whole blood AcAc concentrations significantly decreased by 51% ($p = 0.018$) within 30 days. The concentration of AcAc in fasting and postprandial plasma was notably higher than that of whole blood and red blood cells.
Discussion: Our data demonstrates that plasma for AcAc analysis can be stored for longer than previously suggested provided that plasma is deproteinised and stored at −80 °C.

1. Introduction

Ketone bodies are synthesised and secreted by the liver, when glucose is not readily available, to be utilised peripherally as an alternate energy source [1]. The principal drivers of ketone body production, in healthy individuals, are low plasma insulin concentrations and high plasma non-esterified fatty acid (NEFA) concentrations [2,3]. In healthy adults, in the fasting state, plasma NEFA concentrations are elevated due to intracellular lipolysis of adipose tissue triacylglycerol (TG) being activated [4]. In the transition to the fed state, intracellular lipolysis adipose tissue TG is suppressed by insulin and plasma NEFA concentration decrease [4] leading to less substrate availability for ketone body production [5].

Three ketone bodies are synthesised from acetyl-CoA in the mitochondria of human hepatocytes: acetoacetate (AcAc), 3-hydroxybutyrate (3HB), and acetone, with the latter being less abundant [1]. The process of ketogenesis is dependent on acetyl-CoA forming acetoacetyl CoA, which is converted to 3-hydroxy-3-methylglutaryl CoA (HMG CoA), which is cleaved by HMG CoA lyase to generate AcAc [1,6]. AcAc is reduced to 3HB in a NAD^+/NADH-coupled near equilibrium reaction by 3HB dehydrogenase, in which the equilibrium constant favours 3HB formation [6]. The formation of acetone is produced nonenzymatically by decarboxylation of AcAc [6]. The concentration of ketone bodies in the blood increases during fasting or prolonged exercise and is decreased on the consumption of a mixed meal [1,2,6,7]. Fasting blood concentrations in healthy adults (aged 21–60 years) are reported to be 10–160 μmol/l for AcAc and 5–335 μmol/l for 3HB [8]; notably higher concentrations (1–2 mmol/l) occur in individuals experiencing ketosis [1]. An important route of disposal for fatty acids entering the liver is β-oxidation and 3HB is often used as a proxy marker of hepatic fatty acid oxidation [3,5,9,10]. 3HB modulates the mitochondrial redox potential in the liver [6]. The ratio of arterial 3HB/AcAc can be used as a surrogate marker of the mitochondrial redox state, as the ratio of NADH/NAD^+ cannot be measured directly [11]. In the fasting state the 3HB/AcAc ratio is reported to be 1:1–3:1, but may be higher (6:1) with prolonged fasting, in healthy individuals [1]. High blood 3HB concentrations can arise when there is a displacement of equilibrium, i.e. when ketoacidosis occurs, resulting in very low blood concentrations of AcAc [12]. The correct balance between oxidative and reductive events (redox state) is critical for normal cell function and alterations in the hepatic redox state are suggested to be important in the development of liver disease; oxidative stress is considered a major pathogenic event in several liver diseases, including the progression of steatosis (fatty liver) to non-alcoholic steatohepatitis (NASH) [13–16]. Thus, measuring the 3HB/AcAc ratio may be a useful biomarker in the early detection of liver disease development and progression and require exploration.

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http://dx.doi.org/10.1016/j.cca.2014.03.033
0009-8981/

Blood and/or plasma concentrations of 3HB appear to be measured more often than AcAc which may be due to the long term stability of 3HB [17]. 3HB has been reported to have good storage stability as it is a stable analyte in whole blood, plasma and serum [17]. In contrast, studies measuring plasma AcAc concentrations have reported various degrees of stability, with some suggesting it is preferable to measure AcAc within an hour of blood collection [18] whilst others report plasma AcAc to be stable for 29 days when stored at −80 °C [19]. The concentration of AcAc has been measured in whole blood and plasma [18–20] but a comparison between blood fractions has not been undertaken. The measurement of AcAc has previously been undertaken in untreated and deproteinised plasma [8,18,19,21]. However, the measurement of whole blood or red blood cell AcAc requires samples to be deproteinised: 1) for destruction and removal of cell membranes (releasing any intracellular ketones) and removal of all proteins and 2) because it is not possible to do these types of assays with whole blood or red blood cells as a matrix. Removal of proteins will prevent a continued reduction of AcAc to 3HB in vitro (after sample collection) providing a more accurate reflection of AcAc concentration [22].

Determining the storage stability of AcAc is important as in many situations it is unfeasible for samples to be measured on the day of blood collection and it can be more cost and time effective to run samples in batches at a later date. Therefore the aims of our study were to: 1) compare the storage stability of AcAc in the different blood fractions, and 2) determine the postprandial concentration of AcAc in whole blood, plasma and red blood cells.

2. Subjects

Participants were healthy adults recruited from the Oxford BioBank [23] and the wider Oxford community. All subjects provided written informed consent and studies were approved by the Portsmouth Ethics Committee. Blood was taken from a total of 28 individuals: 5 subjects (age 41 (4.4) years, BMI 24 (1.7) kg/m^2 (mean (SEM))) had blood samples taken that were used to determine the stability of AcAc over time and 23 individuals took part in a study investigating postprandial ketone body metabolism in different blood fractions; baseline characteristics of the latter group are presented in Table 1.

3. Methods

Blood fractions were prepared based on the method described by Humphreys and Frayn [24] for the preparation of perchloric extracts of blood, adapted from Williamson et al. [12]. Briefly, blood was taken from the antecubital vein into heparinised syringes (Sarstedt, Leicester, UK) and mixed by gentle inversion before placing on ice. All three blood fractions were deproteinised with perchloric acid in order for the analysis to be performed using the same methodology on the biochemical analyser.

An aliquot of whole blood was added to a pre-weighed tube containing ice-cold perchloric acid (PCA) (117. 6 g/l (VWR international 101754 'AR' grade PCA)) at a 1:2 ratio and vortexed thoroughly then placed on ice. Plasma was obtained by the centrifugation of the remaining blood (2500 ×*g*, 10 min) at 4 °C. An aliquot was then mixed with ice-cold PCA as described above. After the removal of plasma, the remaining packed red blood cells were washed using a modification of a previously described method [25]. Briefly, red blood cells were washed twice in NaCl (9 g/l) and after centrifugation (2500 ×*g*, 10 min) the top layer and buffy coat were discarded and the remaining red blood cells were mixed with ice-cold PCA as described above.

Deproteinised samples were centrifuged (12,500 ×*g*; 10 min, 4 °C) and the supernatant was transferred to new tubes. Samples were then 'neutralised' in a ratio of 300 μl of sample to 120 μl ice-cold neutralising solution (0.5 mol/l $KHCO_3$, 0.5 mol/l K_2CO_3) and vortexed. Samples were left uncapped on ice for 20–30 min, where the pH was checked to be 8.0, vortexed, and centrifuged (685 ×*g*; 5 min) and the supernatant was transferred to new tubes. For simplicity the term 'neutralised' is used throughout to refer to samples at pH 8.0. All samples were analysed using an ILab 650 clinical chemistry analyser (Instrumentation Laboratory Ltd. UK, Warrington, Cheshire, UK).

3.1. Stability of AcAc in neutralised deproteinised blood fractions over 24 h at 4 °C and −80 °C

The neutralised supernatant was aliquoted into three tubes. One aliquot was stored overnight at 4 °C, one overnight at −80 °C and the third analysed immediately on the ILab 650. The following day the samples from −80 °C were thawed and analysed with samples from 4 °C for AcAc on the ILab 650.

3.2. Stability of AcAc in deproteinised blood fractions in long term storage at −80 °C

Neutralised samples were aliquoted to nine replicates per sample and pooled samples were made for each blood fraction from the remaining un-neutralised supernatant then neutralised as above and aliquoted into nine replicates. All samples except the ones to be analysed that day (day 0) were then stored at −80 °C. On subsequent days after blood collection (days 0, 2, 3, 7, 14, 30, 60 and 90) samples were thawed, centrifuged (685 ×*g*; 5 min) and analysed on the ILab 650.

3.3. Stability of AcAc in PCA

One acid deproteinised aliquot of each sample was kept at 4 °C overnight before being prepared as above and aliquoted into seven replicates per sample. A pooled sample was made for each blood fraction from the remaining un-neutralised supernatant. These were neutralised as previously described and aliquoted into seven replicates. These samples were stored at −80 °C and analysed on the ILab 650 alongside samples prepared for the study described above on days 2,3,7,14,30, 60 and 90 after blood collection.

3.4. Changes in postprandial insulin, NEFA, AcAc and 3HB

Subjects attended the clinical research unit after an overnight fast and baseline blood samples were taken (time 0) prior to the subjects being fed a standard mixed test meal consisting of 40 g Rice Krispies (Kellogg, Manchester, UK), 200 g skimmed milk and a chocolate milkshake containing 40 g of olive oil as previously used [9]; at 360 min subjects were given a drink containing 75 g of glucose. Blood samples were collected regularly over the study period at 0, 60, 120, 180, 240, 300, 360, 390 and 420 min.

Whole blood, plasma and red blood cell samples were prepared as described above; however, red blood cells were mixed with the ice-cold PCA without prior washing. All samples were kept at 4 °C overnight and neutralised the following day as previously described. AcAc was analysed either immediately on the ILab 650 or stored at −80 °C to be

Table 1
Subject characteristics for the postprandial study.

Males/females (n)	12/11
Age (y)	46.0 (1.3)
BMI (kg/m^2)	27.5 (0.5)
Fasting plasma concentrations	
NEFA (μmol/l)	441.1 (30.7)
AcAc (μmol/l)	40.1 (3.6)
3HB (μmol/l)	60.4 (6.9)
Insulin (mU/l)	11.6 (1.2)
Glucose (mmol/l)	5.1 (0.2)

Data presented as mean (SEM). Abbreviations: BMI, body mass index; NEFA, non-esterified fatty acids; AcAc, acetoacetate; and 3HB, 3-hydroxybutyrate.

analysed at a later date. The remaining un-neutralised supernatant was stored at −80 °C for analysis of 3HB on the ILab 650 [24].

3.5. Biochemical analysis of samples

Plasma NEFA concentrations were determined enzymatically using a biochemical analyser (ILab 600) and plasma insulin concentrations were determined by radioimmunoassay (Linco Research, St. Charles, MO) as previously described [26].

The AcAc method was adapted for the ILab 650 from Williamson et al. [12]. The AcAc assay was calibrated using standards (0, 12.5, 25 and 50 μmol/l) of lithium acetoacetate diluted in PCA (66.65 g/l (VWR international 101754 'AR' grade PCA)). Standards (300 μl) were treated as the samples above, i.e. mixed with 120 μl ice-cold neutralising solution, vortexed, left on ice for 20–30 min, and centrifuged (685 ×*g*, 5 min) and the supernatant was transferred to new tubes. The AcAc assay reagent was made up just before analysis and contained: 0.1 mol/l sodium phosphate buffer (pH 7.0), 0.5% NADH, ascorbic acid (35 mg/ml) and 3HB dehydrogenase (5 mg/ml Roche) in 100:1:2:2.5 ratios. For this analysis on the on the ILab 650, 40 μl of sample and 140 μl of reagent were used giving a pH of 7.0.

For 3HB the ILab 650 analyser was calibrated using standards (0, 100 and 200 μmol/l) from a 40 mmol/l stock of 3HB diluted in PCA (66.65 g/l (VWR international 101754 'AR' grade PCA)). 3HB assay reagent one consists of a Tris hydrazine buffer (pH 8.5) containing 2 g/l buffer of NAD. Reagent 2 is a 1:4 dilution of 3HB dehydrogenase (Roche 5 mg/ml) in 0.2 mol/l Trizma base which was made up just before analysis.

The coefficient of variation (CV%) for the analysis of AcAc in the samples used in the stability study, was 20.4% for a concentration of 12.7 μmol/l and 9% for a concentration of 45 μmol/l. The detection limit of the biochemical analyser for AcAc analysis was 5 μmol/l and reproducibility below this was variable and unreliable. For samples with low concentrations (<5 μmol/l) the change in absorbance was less than the 0 standard and in some cases a negative result was given. For statistical analysis the raw values (positive and negative) were used, and any data presented as a negative value represents concentrations of <5 μmol/l that could not be measured accurately or reliably.

4. Statistical analyses

IBM SPSS Statistics (IBM SPSS products, Chertsey, UK) was used for statistical analysis and data is presented as a mean (SEM). Due to calibration issues with the ILab 650 on day 3 of the stability study, there was a notable decrease in AcAc concentration in all three fractions, therefore the data, although presented, has been excluded from statistical analysis. Repeated measures analysis of variance was used to analyse changes in plasma insulin and NEFA over time, AcAc and 3HB concentrations between different blood fractions with time and blood fraction as factors. Linear regression analysis was used to analyse stability of AcAc concentration within a single blood fraction with time and blood fraction as independent and dependent variables. A value of $p < 0.05$ was used to indicate significance.

5. Results

5.1. Stability of AcAc at 4 °C and −80 °C over 24 h

Plasma AcAc concentrations were significantly higher than whole blood ($p < 0.001$) and red blood cells ($p = 0.003$) at time 0 h and after 24 h of storage at either 4 °C or −80 °C (Fig. 1). There was minimal change in plasma AcAc concentrations when deproteinised samples were measured immediately or stored at 4 °C or −80 °C overnight prior to analysis (Fig. 1). In contrast, the AcAc concentration of whole blood and red blood cells was found to be more variable, but not significantly different under the same conditions ($p = 0.984$ plasma, $p = 0.698$ whole blood, and $p = 0.401$ red blood cells, regression analysis over time) with negative values representing measurement variability where concentrations were very low (<5 μmol/l).

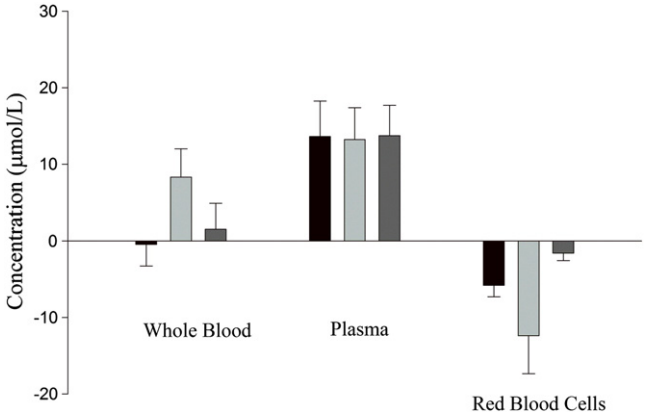

Fig. 1. Concentration of AcAc when measured immediately (■) and stored at 4 °C (▭) or −80 °C (▬) for 24 h in whole blood plasma and red blood cells. There is minimal change in the concentration within the plasma fraction ($p = 0.984$) with non-significant changes in whole blood ($p = 0.698$) and red blood cell fractions ($p = 0.401$).

5.2. Stability of AcAc in long term storage at −80 °C

The AcAc concentration was significantly different between plasma and whole blood ($p = 0.004$), plasma and red blood cells ($p < 0.001$), and whole blood and red blood cells ($p < 0.001$) across the 90 day period, although from 60 days a decline in concentration in all three fractions was noted (Fig. 2A). From days 0 to 30 minimal change was noted in plasma AcAc concentrations (130.0 (19.4) μmol/l (mean (SEM)) day 0 to 129.5 (20.8) μmol/l day 30, $p = 0.647$) and between days 0 and 60 there was a non-significant ($p = 0.244$) decrease in AcAc concentration; however, over the 90 day period plasma AcAc concentrations significantly decreased ($p = 0.023$) (Fig. 2A). In contrast, by the 30th day whole blood AcAc concentrations had significantly decreased (81.3 (19.3) μmol/l day 0 to 40.5 (3.8) μmol/l day 30, $p = 0.018$) (Fig. 2A). Although red blood cells had a lower concentration of AcAc, compared to the other two fractions and greater measurement variability, notably between days 3 and 14 when concentrations were very low (<5 μmol/l), there was no significant change ($p = 0.266$) in red blood cell concentration of AcAc between days 0 and 90 (Fig. 2A).

5.3. Stability of AcAc in PCA

We assessed whether plasma, whole blood and red blood cell samples could be left deproteinised without being neutralised for 24 h at 4 °C. There was very little difference in AcAc concentration in plasma for the two different preparations of the sample: 82.0 (10.9) μmol/l neutralised immediately and 75.6 (11.3) μmol/l neutralised after 24 h ($p = 0.776$) (Fig. 2B). Although there was no significant difference in AcAc concentration between the two preparations for whole blood ($p = 0.142$) and red blood cells ($p = 0.123$) the difference in these two fractions was greater compared with that in the plasma fraction (whole blood: 49.9 (11.0) μmol/l neutralised immediately and 33.0 (11.3) μmol/l neutralised after 24 h, red blood cells: 4.8 (17.9) μmol/l neutralised immediately and −22.3 (29.6) μmol/l neutralised after 24 h) (Fig. 2B).

5.4. Postprandial plasma insulin and NEFA concentrations

After consumption of a mixed test meal, plasma insulin concentrations increased, with a peak at 60 min and then decreased until 360 min when the second meal (glucose drink) was given, causing a very brisk increase ($p < 0.001$, repeated measures over time) (Fig. 3A). In contrast, consumption of the meal resulted in a rapid decrease in plasma

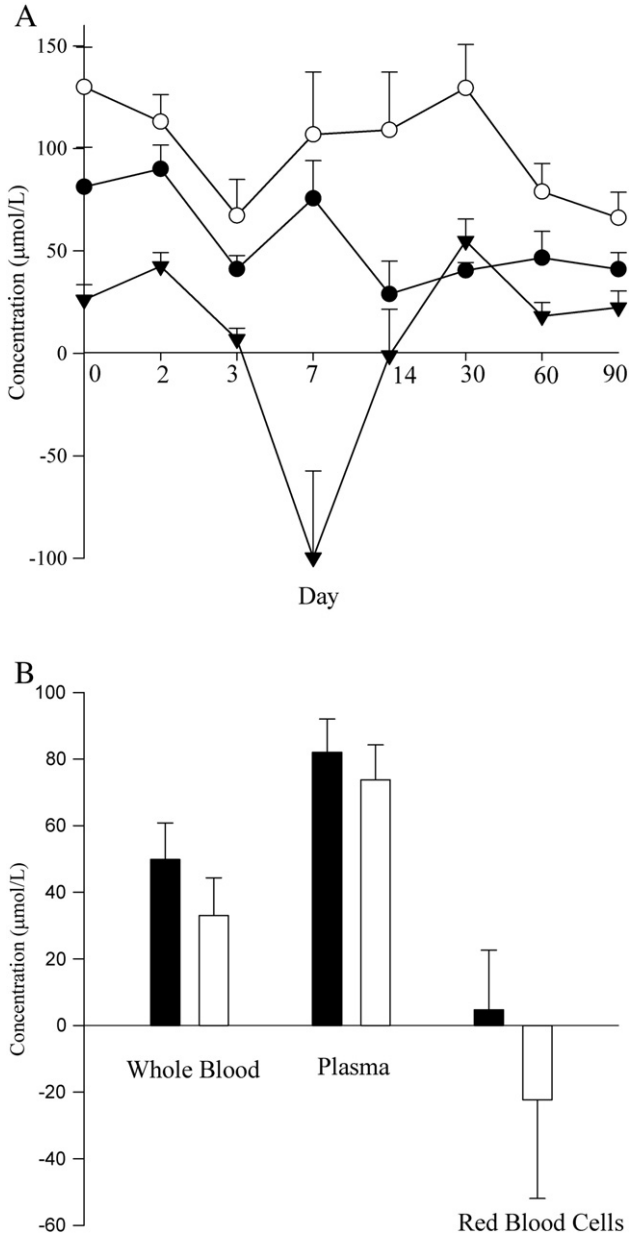

Fig. 2. Stability of AcAc when stored at −80 °C. A) Concentration of AcAc over 90 days when stored at −80 °C in whole blood (●), plasma (○) and red blood cells (▼). Stability and concentration of AcAc were greater in plasma compared to whole blood ($p = 0.004$) and red blood cells ($p < 0.001$). B) Deproteinised whole blood, plasma and red blood cell AcAc concentrations did not differ over 90 days when neutralised immediately (■) or stored at 4 °C overnight (□) prior to neutralisation.

NEFA concentrations until around 120 min when there was a steady rise until 360 min with consumption of the glucose drink resulting in a rapid decrease ($p < 0.001$, repeated measures over time) (Fig. 3B).

5.5. Postprandial AcAc

In line with the stability study, the concentration of AcAc in plasma in fasting samples was consistently higher; this pattern was maintained over the postprandial period with the concentration being almost double than that in whole blood (Fig. 3C). With feeding there was a notable decrease in AcAc concentration between 0 and 120 min in all three blood fractions followed by an increase until 360 min when the second meal was consumed (Fig. 3C). Consumption of the glucose drink (second meal) caused a notable decrease in the concentration of AcAc in all three blood fractions, with concentrations at 420 min being similar to those at fasting (time 0) (Fig. 3C). The changes in AcAc reflect the changes in plasma NEFA concentrations (Fig. 3B). Although the plasma concentration of AcAc was significantly higher than that of whole blood ($p < 0.001$) there was no significant difference in the postprandial pattern over time between blood fractions ($p = 0.077$). When comparing the postprandial pattern of red blood cells there was a significant difference with plasma ($p < 0.001$) and whole blood ($p = 0.018$) (Fig. 3C).

5.6. Postprandial 3HB

The fasting concentrations of 3HB were similar in plasma and whole blood, 60.4 (6.9) μmol/l and 60.2 (6.0) μmol/l respectively, but were lower in red blood cells (45.9 (5.1) μmol/l). After consumption of the test meal 3HB concentrations in all blood fractions decreased until 120–180 min when they started to increase (Fig. 3D). After the second meal (360 min) the concentration of 3HB decreased rapidly with the concentration at 420 min being the same as fasting (time 0). The time course response of 3HB is in line with the postprandial changes in plasma NEFA (Fig. 3B). When whole blood and plasma 3HB concentrations were compared a significant difference ($p = 0.008$) over the postprandial time course was observed. Although the concentrations of 3HB in whole blood and plasma were similar up to 120 min there was then a notable difference ($p < 0.001$) in the postprandial pattern with plasma increasing prominently until 360 min then decreasing rapidly compared to whole blood (Fig. 3D). These differences in 3HB concentrations were also observed when comparing plasma with red blood cells ($p < 0.001$) and whole blood with red blood cells ($p = 0.001$) (Fig. 3D). Not only was the concentration of 3HB much lower in red blood cells compared to the other two fractions, but a lower response to feeding was found in this fraction.

6. Discussion

The concentration of 3HB in blood or plasma is typically measured more often than AcAc, which may be due to its reported long term storage stability. We investigated the effect of storage at 4 °C and −80 °C to determine the storage stability of AcAc in deproteinised whole blood, plasma and red blood cell samples. We found the plasma concentration of AcAc to be consistently and notably higher and to have greater storage stability over 60 days compared to whole blood, where significant changes were observed within 30 days. Our results clearly demonstrate that AcAc in plasma is not as labile as previously reported.

The storage stability of AcAc has been investigated by only a few and these studies have used plasma/serum stored for periods of 24 h–40 days [18,19]. It was found that the AcAc concentration of untreated (non-deproteinised) plasma/serum when stored at −20 °C significantly decreased within seven days but samples stored at −80 °C were stable up to 29 but not 40 days; notably 3HB was stable over the 40 day period [19]. The results of the present study demonstrate that plasma, not whole blood or red blood cell AcAc concentrations are stable for 60 days when stored at −80 °C. A plausible reason as to why we found plasma AcAc concentrations to have longer storage stability than the study of Fritzsche et al. [19] is we deproteinised samples with PCA immediately after collection and then neutralised (to pH 8.0) samples prior to storage at −80 °C. Deproteinisation of blood/plasma samples has previously been recommended for ketone body analysis [22]. The improved stability we found, compared to others, may in part be explained by deproteinisation removing the enzyme 3HB dehydrogenase, thus AcAc is not being continually reduced to 3HB after sample collection.

Our study did not compare deproteinised with non-deproteinised plasma; however, Galan et al. [21] reported no difference in AcAc concentration if samples were immediately deproteinised (and not neutralised) or left non-deproteinised prior to analysis, although storage time was not specified. For the analysis of AcAc, samples need

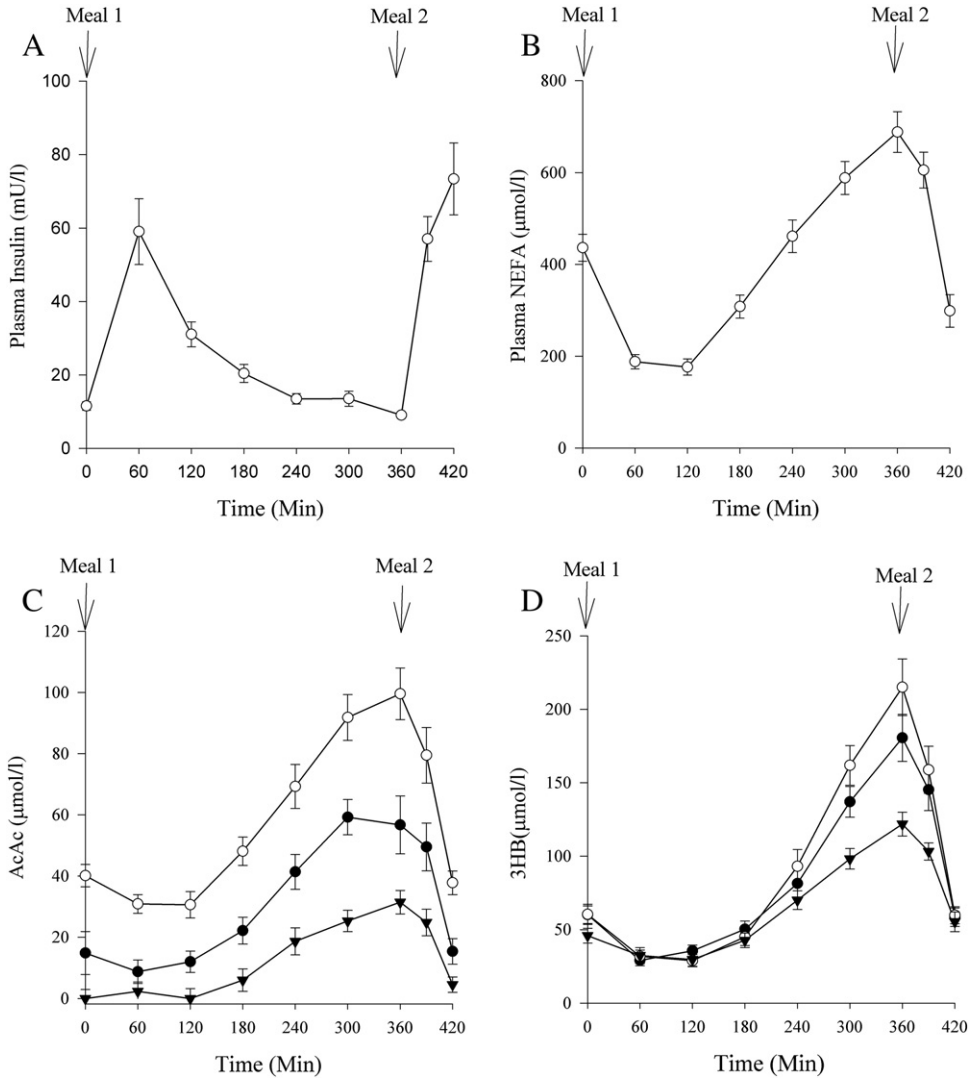

Fig. 3. Concentrations of plasma insulin, NEFA and blood AcAc and 3HB. A) Plasma NEFA concentration (○) over a postprandial period. B) Plasma insulin concentration (○) over a postprandial period. C) AcAc concentrations in whole blood (●), plasma (○) and red blood cells (▼) over a postprandial period. The concentration of AcAc is greater in plasma than whole blood ($p < 0.001$) and red blood cells ($p < 0.001$) over the fasting and postprandial periods. D) 3HB concentrations in whole blood (●), plasma (○) and red blood cells (▼) over a postprandial period. 3HB concentration was similar in plasma and whole blood in fasting but higher in plasma than whole blood ($p = 0.008$) and red blood cells ($p < 0.001$) in the postprandial state. Meal 1 is a mixed test meal and Meal 2 is a glucose (75 g) drink.

to be at pH 7.0 with excess NADH [27] which is achieved when neutralised samples are mixed with the AcAc reagent containing NADH on analysis. We assessed the effect of storing samples overnight at 4 °C in PCA or in neutralised PCA and found no difference in AcAc concentration within the respective blood fractions, with either protocol. Additionally, storing neutralised deproteinised blood samples at either 4 °C or −80 °C overnight had no effect on the concentration of AcAc. Taken together, these findings demonstrate, that contrary to previous suggestions AcAc is not as labile which may be explained by the lack of enzyme activity in deproteinised blood fractions. For the reaction of AcAc to 3HB to go to completion pH 7.0 is needed; untreated plasma is at pH 7.0 therefore the reaction could still be occurring when stored; however, samples stored at pH 8 are less likely to follow this route due to a lack of enzyme activity.

To our knowledge, the concentrations of AcAc and 3HB have not previously been compared simultaneously across different blood fractions or in different nutritional states. We found the plasma concentration of AcAc to be consistently higher than that in whole blood and red blood cells in fasting and across the postprandial period; differences in plasma, whole blood and red blood cell 3HB concentration were not as evident until later in the postprandial period. The similarity in 3HB concentration between the blood fractions is an interesting observation. It is possible that at lower concentrations (≤50 μmol/l) differences between blood fractions are more subtle and difficult to detect. We measured red blood cell 3HB to try and determine if 3HB was present. Although red blood cells had the lowest concentration of 3HB (and AcAc) and whilst this may be a true representation of the concentration of these ketone bodies, we cannot exclude the possibility that there were small amounts of plasma contamination, as red blood cell samples were not washed for the postprandial study and may not have been sufficiently washed for the stability study; in support of this plasma and red blood cell postprandial AcAc and 3HB follow a similar time course pattern.

When measured in the fasted state, blood concentrations of AcAc and 3HB are reported to be up to 160 μmol/l and 335 μmol/l, respectively [8]; which is notably higher than what we found in the early postprandial period (i.e. 120 min after meal consumption). The decrease in the concentration of AcAc and 3HB from a fasted to fed state can be explained by the consumption of the mixed test meal causing an increase in plasma insulin levels and a decrease in plasma NEFA concentrations; the latter being the primary substrate for ketogenesis. In the present study, AcAc and 3HB started to increase after 180 min, which may be explained by

an increase in plasma NEFA concentrations, leading to more fatty acids entering the liver that could be used for ketogenesis [5]. We report that the time course response for 3HB is in line with previous work [9,28].

The measurement of AcAc and 3HB in blood has the potential to provide informative data regarding metabolic processes in the human liver. The redox state of the liver has been implicated to be important in liver disease development and progression; for example progression from steatosis to NASH [13–16]. As the ratio of hepatic NADH/NAD^+ cannot be measured directly measuring the ratio of 3HB/AcAc as a surrogate marker of the mitochondrial redox state [11] may prove to be a useful marker for tracking liver disease development and progression. Plasma AcAc and 3HB can be measured using commercially available assays, such as the Bioassay EKBD 100 EnzyChrom ketone body assay kit, which has a detection limit of 120 μmol/l and recommends samples to be stored at −80 °C and measured within 29 days. However, in healthy individuals after an overnight fast the concentration of blood AcAc is 10–160 μmol/l [8], thus it is likely that the concentration of AcAc in fasting and postprandial blood samples, may be below the level of detection in a commercially available kit. By measuring the concentration of AcAc in plasma, whole blood and red blood cells using a method developed 'in house' we have been able to determine small differences across time and between people, as the detection limit of our assay is 5 μmol/l, using a 40 μl sample volume. Low concentrations of blood AcAc can be detected with the quality of the data being dependent on the condition of the optics of the clinical analyser. We measured whole blood, plasma and red blood cell 3HB concentrations using a method developed in house [24] but have found good agreement between our method and that of the commercially available Randox kit for plasma 3HB. Taken together, our data demonstrate that contrary to previous reports, AcAc concentrations in plasma remain stable once deproteinised and neutralised and stored at −80 °C for up to 60 days.

Author contributions

CAM, CP, SMH and LH designed the research; CAM conducted the study; CAM and SMH conducted the statistical analysis; LH and CAM wrote the paper; CP and SMH provided critical revisions to the manuscript; LH had primary responsibility for the final content; and all authors read and approved the final manuscript.

Conflict of interest

No author has any disclosures to declare.

Funding

The research was funded by the British Heart Foundation (FS/11/18/28633). LH is a British Heart Foundation Intermediate Fellow in Basic Science. CP is a recipient of a Postdoctoral Scholarship from the Henning and Johan Throne-Holst Foundation.

Acknowledgements

The authors thank Marjorie Gilbert for her help in sample analysis; Louise Dennis, Jane Cheeseman, Sue Beatty and Rachel Craven-Todd for their expert help in taking blood samples; and the participants in the study.

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