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Chapter 6

The influence of ketoacids on plasma creatinine assays in diabetic ketoacidosis

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Abstract

Background: Plasma creatinine can be elevated in diabetic ketoacidosis (DKA), due to ketoacid interference with the creatinine assay. This has been reported for the alkaline picrate (Jaffé) methods. Enzymatic assays lack this interference, when ketoacids are added to plasma samples in the laboratory. We performed a clinical study comparing enzymatic and alkaline picrate assays with a high performance liquid chromatography (HPLC) method.

Methods: Nine patients who experienced 10 episodes of DKA were included in the study. Blood samples were drawn before, during and after standard in-hospital treatment. Plasma creatinine was measured with two dissimilar enzymatic assays (creatininase PAP+ and creatinine iminohydrolase Serapak), a kinetic alkaline picrate method (Jaffé) and an HPLC procedure. Acetoacetate and β-hydroxybutyrate were analyzed by enzymatic methods.

Results: At presentation, the Jaffé assay gave falsely high values of plasma creatinine (median 99 μmol/l), in contrast to the PAP+ (median 60.5 μmol/l) and HPLC assays (median 67.5 μmol/l). This positive error decreased during treatment. This was due to a decrease in acetoacetate, as the positive error by the Jaffé method correlated with the acetoacetate concentration (r = 0.79, P < 0.0001). In the multiple regression analysis, β-hydroxybutyrate caused no additional interference by the Jaffé assay, confirmed by in vitro experiments. Analysis of agreement showed that the difference between PAP+ and HPLC creatinine was -4.6 ± 3.0 μmol/l (mean ± SD) and 2.0 ± 5.3 μmol/l between Serapak and HPLC. This was statistically significant, but clinically negligible.

Conclusion: Acetoacetate caused severe interference of the alkaline picrate (Jaffé) assay, which might influence therapeutic decisions at the start of diabetic ketoacidosis. Enzymatic assays lack this interference.
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Introduction

In patients presenting with diabetic ketoacidosis (DKA) the plasma creatinine concentration can be elevated due to three causes. First, diabetic patients may have an elevated plasma creatinine due to the presence of diabetic nephropathy. Second, dehydration may develop in the course of DKA, because of osmotic diuresis of glucose and ketoacids [1]. Finally, interference of ketoacids with the plasma creatinine assay can result in a falsely high plasma creatinine concentration [2-4]. In acute situations it may be difficult to distinguish these three factors, especially when information on a previous plasma creatinine concentration is not available. Plasma creatinine is often elevated in case of dehydration. This is especially relevant in older patients with moderate or poor cardiac function possibly in combination with impaired renal function, who should be rehydrated carefully, opposed to young patients presenting in a comatose state.

In case of DKA the plasma creatinine concentration is also used as a measure of the glomerular filtration rate. A stable plasma creatinine can be applied for calculation of the creatinine clearance, either by using urine creatinine concentration and urine volume per unit of time or in a variety of formulas by estimating urinary creatinine excretion from age, gender and body weight [5-9]. In vitro, the alkaline picrate - or Jaffé - method leads to a falsely increased plasma creatinine value due to non-creatinine chromogens, such as acetoacetate [10]. Protein precipitation and dialysis have been used to decrease these interferences [2,3]. β-Hydroxybutyrate has been reported to cause no interference, while acetone and glucose interfere to a lesser extent [2,4,11]. The time dependent or kinetic alkaline picrate method has been advocated as an improvement for interferences, compared to the end point analysis [12-15]. However, even negative interferences induced by acetoacetate have been reported with the kinetic method [16]. Enzymatic methods are likely to have less interference than the alkaline picrate method. Automated enzymatic creatinine assays were introduced in clinical medicine more than ten years ago, but still are not generally used because of financial reasons [17-20]. The ion-exchange high performance liquid chromatography (HPLC) procedure with ultraviolet detection at 234 nm has been proposed as a candidate reference method, because results obtained with it are similar to the definitive isotope-dilution mass spectrometry [21,22].

Laboratory studies, using "spiked" plasma samples i.e. with added acetoacetate, have shown that the enzymatic assay is not susceptible to acetoacetate interference [17,18,20,23]. However, in patients with DKA who have a variety of metabolic disturbances such as hyperglycaemia and
dehydration, ketoacidosis and sometimes hyperchloeaemic acidosis, the accuracy of the enzymatic method is not known. The aim of our study was to investigate the degree of interference of ketoacids in patients during and after DKA in the clinical situation. Therefore the alkaline picrate and enzymatic assays were compared with an HPLC method.

Material and methods

Patients
From december 1997 to december 1998 nine patients presenting with 10 episodes of DKA to the Department of Medicine, Academic Medical Center, Amsterdam were included in the study. Mean age was 36 years, four patients were male. Two patients had de novo diabetes, three patients were treated with a subcutaneous insulin pump, the other four with a combination of long-acting insulin at bedtime and regular insulin before meals. Proteinuria was absent in these patients. None of the patients was jaundiced or treated with cephalosporins. The diagnosis of DKA was made in diabetic patients when the following two conditions were present: 1. a high anion gap (≥ 20 mmol/l) metabolic acidosis or an arterial bicarbonate <17 mmol/l and 2. a positive Ketostick in the urine. The patients were treated according to a standard in-hospital protocol with intravenous fluids and insulin until normovolaemia and normoglycaemia without acidosis were reached.

Study protocol
At presentation one arterial and two venous heparinized blood samples were drawn. One sodiumfluoride coated tube was drawn for analysis of ketoacids and put on ice immediately. Ketostick and Albustick (Boehringer Mannheim, Mannheim, Germany) analysis of the urine was performed. The collection of blood samples was repeated at 1, 3 and 6 hours after the start of treatment, and later untill correction of DKA had been obtained. In all patients one extra sampling was done 24 hours after correction of ketoacidosis. The study was approved by the comittee of Medical Ethics of the Academic Medical Center, Amsterdam. Patients were asked to participate in the study within 24 hours after presentation. All patients gave written informed consent.
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**Laboratory methods**

Creatinine was analyzed immediately in heparinized plasma on a Hitachi 747 with the enzymatic PAP+ assay (Phenol/4-Aminoantipyrine, Boehringer Mannheim, Mannheim, Germany). This assay consists of three enzymatic steps starting with creatininas e and a final chromogen coupling reaction. Heparinized plasma was also stored at -20°C and analyzed later by three methods:

-1- The modified or kinetic alkaline picrate - Jaffé - method (Synchron CX3 analyzer, Beckman, U.S.A.). The assay is based on the formation of an orange-red Janovský complex between creatinine and picrate under alkaline conditions. The time between introduction of sample and start of reaction rate absorbance measurement was 25.6 seconds. This is relevant as this lag time partly determines the magnitude of interference.

-2- A second enzymatic creatinine Serapa k assay (Bayer, Tarrytown, USA) on a Beckman Synchron CX-4 analyzer. In this assay the enzymatic step is creatinine iminohydrolase.

-3- An HPLC method after sample ultrafiltration as described by Zwan g and Bleijenberg [24].

Acetoacetate and β-hydroxybutyrate were measured with enzymatic assays (Boehringer Mannheim, Mannheim, Germany). Prior to measurement these samples were deproteinized within 10 minutes after venapuncture with a 1:1 mixture of fluoride plasma and 0.1 N perchloracetic acid and stored at -20°C for three to ten days. In the arterial blood sample pH and P_{CO2} were measured on a blood-gas analyzer (Bayer Diagnostics, Rapid Lab, model nr. 865, Mijdrecht, The Netherlands) and the bicarbonate concentration was calculated with the Henderson-Hasselbach equation. Chloride, urea (Boehringer Mannheim) and glucose (Merck art. nr. 1.12194, Darmstadt, Germany) were analyzed in heparinized plasma on a Hitachi 747. The anion gap was calculated as plasma [Na⁺]-[Cl⁻]-[HCO₃⁻] (normal value in our laboratory: 5.5 - 10 mmol/l). Plasma osmolarity was calculated as 2 x plasma [Na⁺] + [glucose] + [urea] and expressed as mOsmol/kg. Plasma concentration units are given in mmol/l, except for creatinine (µmol/l). We also performed an _in vitro_ experiment, in which an increasing concentration of acetoacetate and β-hydroxybutyrate was added to an aqueous solution of creatinine (100 µmol/l). In this way the _in vitro_ interference of the alkaline picrate assay was tested.

**Statistical analysis**

Data are expressed as means ± SD unless stated otherwise. Statistical analysis was performed using SPSS for Windows, release 7.5. A difference with _P_ <0.05 was considered to be significant. The differences between either two enzymatic or alkaline picrate assays and the HPLC-method...
were tested with the paired t-test. These differences were compared with the ketoacid concentration in a correlation plot. A linear mixed model was performed in SAS, release 6.12, to analyse this correlation for separate patients and the group as a whole. Multiple regression analysis was performed to detect whether, besides acetoacetate, \( \beta \)-hydroxybutyrate or glucose would also contribute to the differences between the alkaline picrate and HPLC creatinine concentration. This was done by stepwise forward selection starting with acetoacetate. Analysis of agreement according to Bland and Altman was done for comparison of the two enzymatic and HPLC-assays \cite{25}.

Table 1 Laboratory investigations (median and range) during treatment of DKA \((n = 10)\). Time 0 shows the values at presentation, time \(>24\) the values 24 hours after correction of ketoacidosis. \(\beta\)-OH-butyrate = \(\beta\)-hydroxybutyrate.

<table>
<thead>
<tr>
<th>Treatment time (hours)</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>6</th>
<th>(&gt;24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.24</td>
<td>7.26</td>
<td>7.31</td>
<td>7.35</td>
<td>7.40</td>
</tr>
<tr>
<td>anion gap (mmol/l)</td>
<td>28</td>
<td>22</td>
<td>15</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>(18-33)</td>
<td>(13-28)</td>
<td>(8-22)</td>
<td>(7-20)</td>
<td>(0-8)</td>
</tr>
<tr>
<td>acetoacetate (mmol/l)</td>
<td>3.4</td>
<td>2.2</td>
<td>1.7</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>(1.3-5.1)</td>
<td>(1.1-3.6)</td>
<td>(0.2-3.0)</td>
<td>(0.0-1.5)</td>
<td>(0.0-0.5)</td>
</tr>
<tr>
<td>(\beta)-OH-butyrate (mmol/l)</td>
<td>5.1</td>
<td>3.4</td>
<td>2.6</td>
<td>1.3</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>(2.7-7.7)</td>
<td>(1.8-6.4)</td>
<td>(0.5-4.5)</td>
<td>(0.0-3.1)</td>
<td>(0.0-1.1)</td>
</tr>
<tr>
<td>glucose (mmol/l)</td>
<td>27</td>
<td>16</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>(13-56)</td>
<td>(5-50)</td>
<td>(2-43)</td>
<td>(7-38)</td>
<td>(6-30)</td>
</tr>
<tr>
<td>osmolality (mOsmol/kg)</td>
<td>301</td>
<td>295</td>
<td>298</td>
<td>284</td>
<td>292</td>
</tr>
<tr>
<td></td>
<td>(281-406)</td>
<td>(278-398)</td>
<td>(278-397)</td>
<td>(270-395)</td>
<td>(276-347)</td>
</tr>
</tbody>
</table>

Results

The results of laboratory investigations before and during the treatment of 10 episodes of DKA are given in Table 1. The time course of the plasma creatinine concentration for the three different methods is shown in Figure 1. The alkaline picrate assay (Jaffé) gave falsely high values of plasma creatinine, in contrast to both the enzymatic PAP+ and HPLC method especially in the first few hours of DKA. The median positive error of the Jaffé assay at the start of treatment
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**Figure 1** Box and whisker-plots of the plasma creatinine concentration during treatment. The alkaline picrate (Jaffé) assay is compared with the enzymatic PAP+ assay (upper panel) and with the HPLC method (lower panel). Treatment time 0 shows the values at presentation, time >24 the values 24 hours after correction of the ketoacidosis. The box represents the 25th and 75th percentile, the line within is the median value. Also the 10th and 90th percentile whiskers are given.

was 64% compared to the enzymatic PAP+ and 47% compared to the HPLC method ($P < 0.0001$). The median enzymatic PAP+ and HPLC creatinine concentrations decreased only slightly during correction of the ketoacidosis (12 and 14.5 µmol/l). This was in contrast to the median Jaffé creatinine, which decreased from 99 to 62 µmol/l ($P = 0.001$ and $P < 0.001$ for the difference in decrease).

**Figure 2** Analysis of agreement according to Bland and Altman [25]. In this analysis the difference between two assays is plotted against their mean for each plasma sample. This was done for the enzymatic PAP+ and HPLC assays. The mean difference is indicated by a drawn line, the limits of agreement (mean ± 2SD and mean ±2SD) are indicated by the dashed lines.
Figure 3  The relationship between the difference of the alkaline picrate (Jaffé) and the HPLC creatinine values against the acetoacetate concentration (○): \( r = 0.79, P < 0.0001 \); the absence of a relationship between the difference of the enzymatic PAP+ and the HPLC creatinine values against the acetoacetate concentration (▲): \( r = -0.01 \), N.S. The dashed line indicates zero difference between two assays.

Analysis of agreement between the enzymatic PAP+ and HPLC creatinine concentrations is shown in Figure 2. The difference between the two methods was -4.6 ± 3.0 \( \mu \text{mol/l} \) \( (P <0.001) \) and was stable over the whole range of plasma creatinine. The mean difference between the enzymatic Serapak and HPLC was 2.0 ± 5.3 \( \mu \text{mol/l} \) \( (P = 0.012, \text{not shown}) \) over the whole range. To analyze whether the positive error of plasma creatinine by the Jaffé assay was due to acetoacetate, the differences between either Jaffé or enzymatic PAP+ creatinine and the HPLC creatinine concentrations were plotted against the acetoacetate concentration (Figure 3). A marked correlation was found between the positive error by the Jaffé assay and the acetoacetate concentration \( (r = 0.79, P < 0.0001) \), whereas the enzymatic PAP+ assay did not show this relationship \( (r = -0.01) \). This was also true for the enzymatic Serapak assay \( (r = -0.11, \text{not shown}) \).

Table 2  Results of forward stepwise multiple regression analysis (50 plasma samples). Dependent variable: Jaffé creatinine concentration minus HPLC creatinine concentration. \( \beta\)-OH-butyrate = \( \beta \)-hydroxybutyrate; SE(B) = standard error of the B-coefficient.

<table>
<thead>
<tr>
<th>Variable</th>
<th>B-coefficient</th>
<th>SE (B)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>8.2</td>
<td>2.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Acetoacetate (mmol/l)</td>
<td>6.3</td>
<td>2.1</td>
<td>0.004</td>
</tr>
<tr>
<td>( \beta)-OH-butyrate (mmol/l)</td>
<td>-0.1</td>
<td>1.3</td>
<td>0.949</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>0.4</td>
<td>0.1</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Table 3 The in vitro influence of acetoacetate and β-hydroxybutyrate on the alkaline picrate - Jaffé - creatinine assay. The ketoacids are dissolved with increasing concentrations in an aqueous solution of creatinine (100 μmol/l).

| Differences in the plasma creatinine concentration determination (μmol/l) | Concentration of acetoacetate and β-hydroxy(=OH)butyrate (mmol/l) |
|---|---|---|---|---|---|
| | 0 | 2.5 | 5.0 | 7.5 | 10.0 |
| acetoacetate | 0 | 13 | 24 | 35 | 45 |
| β-OH-buturate | 0 | 0 | 0 | 0 | 2 |

A correlation was also present when the differences in plasma creatinine were plotted against the β-hydroxybutyrate concentration (r = 0.75, P < 0.0001 for the Jaffé assay, not shown). However, multiple regression analysis showed that besides acetoacetate, only glucose but not β-hydroxybutyrate contributed to the positive error by the Jaffé assay. The B-coefficients are given in table 2. It is shown that without the influence of acetoacetate and glucose the Jaffé assay led to a falsely increased value of plasma creatinine of 8.2 μmol/l, compared to the HPLC method. Each mmol/l of acetoacetate caused an additional increase of 6.3 μmol/l and each mmol/l of glucose an additional increase of 0.4 μmol/l. When β-hydroxybutyrate was removed from the analysis, the standard error of the B-coefficient of acetoacetate and P-value decreased (SE (B) = 0.9, P < 0.001). The in vitro experiment showed that β-hydroxybutyrate did not interfere with the Jaffé assay up to a concentration of 7.5 mmol/l, whereas 1 mmol/l acetoacetate led to an increase of plasma creatinine of 4.5 to 5.2 μmol/l (Table 3).

Discussion

The purpose of this study was to analyse the clinical importance of the interference of ketoacids with various plasma creatinine assays during an episode of DKA. In the literature, most studies investigating ketoacid interference were done in vitro using samples with addition of ketoacids to patient plasma or serum, sometimes in high doses not encountered in clinical DKA. Our study showed that two enzymatic assays - analyzing plasma creatinine in dissimilar enzymatic steps - were similar to the HPLC assay at all stages of DKA. The creatininas e PAP+ assay gave lower values than the HPLC method (mean negative error 5 μmol/l), while the mean creatinine iminohydrolase Serapak assay gave higher values than HPLC (mean positive error 2 μmol/l).
Although these errors were statistically significant, they were negligible from a clinical point of view. Also, the variability of the differences between enzymatic and HPLC values was small.

This is in contrast to the widely used kinetic alkaline picrate or Jaffé method, which led to falsely increased values of approximately 50% at presentation, compared to HPLC. For instance, this led to a plasma creatinine concentration of 152 μmol/L (Jaffé) instead of 107 μmol/l (HPLC) or 102 μmol/l (PAP+) in a severely dehydrated female patient, who had an HPLC creatinine of 51 μmol/l after correction of the ketoacidosis and hydration status. In a normovolaemic female patient, the positive error was even more pronounced: 75 μmol/l (Jaffé) versus 40 μmol/l (HPLC) or 37 μmol/l (PAP+), whereas the final HPLC creatinine was only slightly lower (34 μmol/l). These examples show that the falsely increased values of plasma creatinine using the alkaline picrate (Jaffé) method could have influenced the therapeutic prescription in these patients at presentation.

Previous laboratory studies have shown the inaccuracy of the alkaline picrate assay in the presence of acetoacetate [2-4]. Most of them were published in the 1970s, before the introduction of kinetic modifications of this assay. Later, studies on the kinetic assay showed contradictory results of acetoacetate interference, probably due to the different lag-times between addition of the last reagent and the absorbance measurement of creatinine concentration [11-17,23,26]. The enzymatic assay was shown not to have any interference from acetoacetate [17,18,20,23]. However, the majority of these studies were performed in vitro using “spiked” samples, i.e. with acetoacetate added in a fixed concentration. In clinical studies a surrogate marker for acetoacetate, the anion gap or Ketostick analysis of plasma, was used [4,13,17]. In the present study patient samples were analyzed taken during clinical DKA in patients without diabetic nephropathy. The concentrations of acetoacetate and possible other interfering compounds were beforehand unknown, whereas bilirubin and cephalosporins were excluded as interfering substances. The new generation kinetic alkaline picrate assay showed a relevant positive interference, compared to the HPLC method, which did not occur with two enzymatic assays. This interference was clearly correlated to the acetoacetate concentration. At presentation of DKA, the range of acetoacetate concentration was 1.3 to 5.1 mmol/l and 3.6 and 3.9 mmol/l in the two mentioned examples. The interference was greater than expected from laboratory studies using “spiked” samples up to 20 mmol/l acetoacetate and from our own in vitro experiment [17,18,20,23]. This can be due to acetone in the patient sample. Acetone was not measured, due to its volatile nature and the difficulty to analyse it at presentation. The multiple
regression analysis showed that β-hydroxybutyrate did not cause the positive error and that glucose had influence only in very high concentrations. The *in vitro* experiment also showed no interference of β-hydroxybutyrate. This is in accordance with earlier laboratory studies in which β-hydroxybutyrate did not cause interference in the alkaline picrate method [2,4,11]. One laboratory study showed that each mmol/l of glucose caused a positive error of 0.3 to 0.4 μmol/l of creatinine, which is similar to the estimation in our multivariate analysis [23].

The present study was restricted to patients with clinical DKA. They comprise a minority of hospital admissions. However, Gerard et al. performed a three-month survey study in which they identified 50 ketotic patients, almost 5% of all hospital admissions to the medical department [17]. Diabetes mellitus was the single cause in 20 of them, another 23 where due to ethanol abuse (8 of them had diabetes) and 9 patients were ketotic because of malnutrition in a severe or terminal illness. Plasma creatinine was analyzed with the enzymatic Kodak Ektachem 400 assay and with the alkaline picrate method on Beckman or Technicon Instruments. The mean positive error of the alkaline picrate assay was 124 μmol/l with a range of 35 to 389 μmol/l, in comparison to the enzymatic plasma creatinine assay. No reference method was used. This study illustrates that ketosis is not uncommon in clinical practice and that almost half of the cases are not due to diabetes mellitus. The latter cases will be overlooked, even if the clinician is aware of interference due to DKA. It is likely that most clinicians are not aware of this interference as the latest report in a clinical journal was in 1984 [14].

In conclusion, clinical DKA interferes with a kinetic alkaline picrate assay and not with two different enzymatic plasma creatinine assays, compared with HPLC. The magnitude of this interference can influence the interpretation of the clinical situation in a patient with DKA, which in turn can be important for the management of an episode of DKA. The falsely increased values of the plasma creatinine concentration analyzed with the alkaline picrate assay during DKA are correlated to the acetoacetate concentration, whereas β-hydroxybutyrate causes no additional positive error.

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References


