Re-evaluation of the Plasma Creatinine Determination by the Acidification Method

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Summary

The acidification method for measuring plasma creatinine has been used to determine the role played by bilirubin and ingested drugs, as interfering substances in the Jaffe reaction. This work was made necessary because of earlier reports by some authors that the acidification method for the determination of creatinine is non-specific. The reason given for the non-specificity of this method is that acidification does not only destroy creatinine chromogen but non-creatinine chromogen as well, chief among them being bilirubin.

In this study it was evident that the interfering role of bilirubin is nil; it has no influence on creatinine levels determined by the acidification method, if plasma samples are depro-teinized prior to analysis. This fact is being particularly emphasised as it has not been emphasised in the literature. It was also evident that over a wide pH range (4.5 – 12) bilirubin chromogen measured by the Jaffe reaction remained fairly stable and contributed insignificantly to the creatinine level in solution. Plasma creatinine was completely destroyed after acidification.

Plasma creatinine levels in Ghanaians measured by our method is not significantly different from the true plasma creatinine level in Caucasians measured by more sophisticated methods. The modified acidification method is therefore recommended as a simple, inexpensive and practical method for the routine determination of plasma creatinine.

Key Words: Creatinine determination; non-creatinine chromogen; plasma creatinine

Introduction

The plasma creatinine concentration is commonly used to assess renal function, as the plasma concentration of creatinine reflects to an extent, the kidneys capacity to excrete creatinine, mainly through the process of glomerular filtration. The reliability of this test as a measure of glomerular function is limited by the presence of interfering substances (non-creatinine chromogens) in the plasma, and the fact that some amount of creatinine is secreted by the renal tubules.

It is well known that some substances interfere with the colorimetric determination of creatinine in the plasma, using the Jaffe reaction.\textsuperscript{1-3} For this reason the original Jaffe method has been modified to reduce the influence of interfering substances and hence enhance the accuracy by which creatinine levels in the plasma may be measured. One such modification is the acidification method originally proposed by Slot\textsuperscript{4}. The rationale of this method is that acidification of the reaction mixture containing creatinine resulted in the fading of the colour that is due to creatinine. The remaining colour was then due to substances other than creatinine and such substances were deemed to be more stable than creatinine in an acid medium. These substances are designated non-creatinine chromogens. To the extent that acidification causes com-
plete disappearance of the creatinine chromogen without affecting non-creatine chromogens, the acidification method can be considered as a specific method for creatinine determination. However this method has been challenged and refuted by other workers who claim that acidification also causes destruction of non-creatine chromogens, thereby limiting the specificity of this method for creatinine. While workers have implicated bilirubin as an interfering substance, others have been unable to demonstrate this effect of bilirubin.

This paper presents evidence to support the claim that (1) Bilirubin is not an interfering substance in the Jaffe reaction using the modified method of Slot; (2) this method is fairly specific for creatinine.

Materials and Methods

The effects of time and pH on the colour of the reaction mixture, the role of bilirubin as an interfering agent in the plasma of normal Ghanaians and the interference due to ingested drugs, were studied.

i. Effect of time on creatinine chromogen determination:

Creatinine working solutions having concentrations of 200 μmol/l, 800 μmol/l and 1600 μmol/l were prepared by diluting a stock standard solution containing 2000 μmol/l in 0.1M HCl. The following procedure for deproteinizing plasma samples was followed. Two milliliters of each solution was pipetted into clean, separate test tubes and to each was added 2ml distilled water, 2ml of 5% sodium tungstate and 2ml 0.33M H₂SO₄. The contents of each tube was mixed with a vortex mixer. Since there was no precipitate, there was no need to centrifuge or filter the solutions after the deproteinization process. 2ml aliquots of each solution were transferred into separate test tubes and to each was added 2ml of water and 1ml each of 0.04M picric acid and 0.75M NaOH in sequence. After mixing, the absorbance of each solution was read at 500nm wavelength on a spectrophotometer at the following times: 3, 5, 10, 20, 30, 40, 50 and 60 min. At the end of 60 min 1ml of 0.5M H₂SO₄ was added to each of the solution. The pH of the resulting solution was 1.65. The absorbance was read again after 3, 6 and 10 min at 500nm wavelength.

Linearity between creatinine and absorbance was established for the concentration range 0-2000 μmol/l at 500nm wavelength. 2ml aliquots of each solution were reacted with picric acid and NaOH as above, and the reaction mixture was incubated at room temperature for 40 min. This time was chosen as the optimum time for the resulting colour of the reaction, after which there was little change in the colour intensity with time.

Each solution was analysed in triplicate in all the experiments, and the mean value taken.

ii. The effect of pH on creatinine chromogen:

This effect was studied on two solutions of creatinine at two concentrations: 200 and 400 μmol/l. The procedure was similar to that in section (i) with the following modifications. Sodium tungstate and H₂SO₄ were replaced by water. In addition, the effect of pH on creatinine chromogen was studied at 10 different pH values at each creatinine concentration. The reaction mixture in each tube was incubated at room temperature for 40 min after the addition of alkaline picric acid. At the end of each incubation period the pH of the mixture in each tube was adjusted with glacial acetic acid (1M-17.5M) with the aid of a pH meter. Each tube was allowed to stand for 10 min after the pH of its contents had been adjusted, and the absorbance of the mixture was read at 500nm.

iii. The role of Bilirubin as an interfering agent in the Jaffe reaction:

Bilirubin chromogen was determined at different pH values in order to assess its contribution to the total chromogen in the Jaffe reaction, using bilirubin solutions at three different concentrations: 100, 200 and 400 μmol/l. The procedure here was the same as in section (ii).

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In order to ascertain the effect of bilirubin on plasma creatinine chromogen, the experiment was repeated as above, on plasma samples with known amounts of bilirubin added to them. The following bilirubin solutions in plasma were used: 20, 50, 100, 200 and 400 μmol/l. The resulting mixture was deproteinized following the procedure described under Section (i) of materials and methods. A blank sample containing plasma alone was treated similarly. After deproteinization the mixture was left to stand for 15 min and then centrifuged at 2500 r.p.m. for another 10 min. The supernatant solutions were filtered and treated as in section (i) above. After reading the absorbance of these solutions, each solution was acidified with 0.5M H₂SO₄ as in section (i) and the absorbance read again. From the first reading, the absorbance due to total chromogen was determined and the second reading represented the absorbance due to bilirubin chromogen, i.e. after fading of creatinine chromogen, following acidification.

iv. Effect of pH on mixtures containing creatinine and Bilirubin:

Solutions containing either bilirubin alone or bilirubin and creatinine were used. The solutions contained either 400 μmol/l bilirubin or 400 μmol/l each of bilirubin and creatinine prepared from stock solutions. The procedure here was the same as for section (ii).

v. Interference due to ingested drugs:

This was studied using aqueous solutions of the following drugs: Paracetamol, Chloroquine, Tetracycline, Ampicillin, Indomethacin. These are commonly ingested drugs used in the treatment of bacterial infections and malaria. Others are commonly used analgesic or anti-inflammatory agents. 2ml of known concentrations of each solution was treated as for creatinine by adding 2ml of water and 1ml each of 0.04M picric acid and 0.75M NaOH and reading the absorbance after 40 minutes. Thereafter, decolorisation of the developed yellow colour was achieved by adding 0.5M H₂SO₄ as described earlier. The absorbance was read again after 10 min. The absorbances before and after adding acid were compared with a creatinine standard curve and the results expressed as μmol/l creatinine.

Results

Linearity: The calibration curve for the method used is shown in fig. 1a. There is linearity over the range 0–2000 μmol/l.

Effect of time and pH on creatinine chromogen determination: The colour of the reaction mixture became increasingly intense from the time of adding the alkaline picric acid. (fig. 1b). After 40 min, changes in the colour intensity with time were insignificant. Acidification of the reaction mixture at the end of 60 min reduced the colour intensity to zero within 3 min, irrespective of the initial creatinine concentration in the reaction mixture.

Acid titration of the reaction mixture containing different concentrations of creatinine (200 and 400 μmol/l) gave a characteristic curve (fig. 2a).

At pH greater than 9.0 there was no significant effect of pH on creatinine chromogen. However, as the pH fell from this value, there was a precipitous fading of the colour intensity of the reaction mixture. At pH 6.75–7.0, the colour had completely faded, and the absorbance was reduced to zero, irrespective of the initial creatinine concentration.

Influence of bilirubin on creatinine chromogen determination: The results of the effect of having both bilirubin and creatinine in solution, on the creatinine chromogen concentration are shown in table 1. It is clear that bilirubin in the concentration range 20–400 μmol/l had no effect on the plasma creatinine concentration after deproteinization of the plasma. This is evidenced by a constant absorbance of the reaction mixture despite the presence of varying amounts of bilirubin in solution with creatinine, in the plasma. The effect of pH on the colour due to bilirubin alone in solution
and a solution containing bilirubin and creatinine, after adding alkaline picric acid to the solution are shown in fig 2b.

As can be seen the effect of pH on bilirubin chromogen is negligible regardless of the concentration of bilirubin in solution (fig. 3). It is also clear that when bilirubin is present with creatinine in solution, there is no significant influence of bilirubin on creatinine chromogen over a wide pH range (5–12). As can be seen in fig 2b bilirubin contributed very little to the total chromogen. Acidification of the mixture resulted mainly in fading of the colour due to creatinine in solution.

Interference due to drugs: Chloroquine, paracetamol, tetracycline, indocid, ampicillin, gave positive results when reacted with alkaline picric acid. The concentration of these drugs that resulted in a positive reaction were much greater than the therapeutic levels of these drugs in the blood. The result of the reaction of these drugs with alkaline picric acid is given in table 2 and expressed as equivalent creatinine concentration.

Reproducibility of the method used in this report, was established by repeated measurements of the creatinine concentration of pooled plasma samples. Within–batch coefficient of variation was 3.5% and between–batch coefficient of variation was 3.7%. Mean values of the pooled plasma samples ranged between 91.5 and 92.7 µmol/l, although this range does not reflect the wide range of values for normal plasma levels of creatinine.

Discussion

This study provides evidence to suggest that the acidification method originally described by Slot⁴ for the determination of plasma creatinine is dependable provided that the plasma
Table 1. Effect of Exogenous Bilirubin on Plasma Creatinine Concentration

<table>
<thead>
<tr>
<th>Vol. of Plasma</th>
<th>Concentration of Bilirubin μmol/l</th>
<th>ABSORBRANCE Total</th>
<th>WITH Acid</th>
<th>CONCENTRATION μmol/l Creatinine Total</th>
<th>True Creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ml</td>
<td>0</td>
<td>0.144</td>
<td>0.026</td>
<td>119.3</td>
<td>97.2</td>
</tr>
<tr>
<td>1 ml</td>
<td>20</td>
<td>0.144</td>
<td>0.024</td>
<td>119.3</td>
<td>99</td>
</tr>
<tr>
<td>1 ml</td>
<td>50</td>
<td>0.148</td>
<td>0.028</td>
<td>121.9</td>
<td>97.1</td>
</tr>
<tr>
<td>1 ml</td>
<td>100</td>
<td>0.144</td>
<td>0.025</td>
<td>119.3</td>
<td>97.2</td>
</tr>
<tr>
<td>1 ml</td>
<td>200</td>
<td>0.146</td>
<td>0.026</td>
<td>119.3</td>
<td>97.2</td>
</tr>
<tr>
<td>1 ml</td>
<td>400</td>
<td>0.148</td>
<td>0.028</td>
<td>121.9</td>
<td>97.1</td>
</tr>
</tbody>
</table>

Bilirubin causes no interference if the plasma samples undergo deproteinization. The absorbance readings are similar despite the different quantities of bilirubin added to the plasma samples.

Table 2. Drug Interference in the Jaffe Reaction

<table>
<thead>
<tr>
<th>Drug</th>
<th>Absorbance</th>
<th>Creatinine Equivalent μmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>With Acid</td>
</tr>
<tr>
<td>Paracetamol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;20 mg/ml&quot;</td>
<td>0.025</td>
<td>0.080</td>
</tr>
<tr>
<td>Chloroquine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;300 μg/ml&quot;</td>
<td>0.004</td>
<td>0.000</td>
</tr>
<tr>
<td>&quot;40 μg/ml&quot;</td>
<td>0.135</td>
<td>0.164</td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;100 μg/ml&quot;</td>
<td>0.105</td>
<td>0.085</td>
</tr>
<tr>
<td>&quot;10 μg/ml&quot;</td>
<td>0.007</td>
<td>0.000</td>
</tr>
<tr>
<td>Indocid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;5 mg/ml&quot;</td>
<td>0.355</td>
<td>0.048</td>
</tr>
<tr>
<td>&quot;600 μg/ml&quot;</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>&quot;6 μg/ml&quot;</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Ampicillin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;25 mg/ml&quot;</td>
<td>0.039</td>
<td>0.004</td>
</tr>
<tr>
<td>&quot;250 μg/ml&quot;</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>&quot;5 μg/ml&quot;</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

sample is deproteinised prior to analysis for creatinine. It also provides evidence that bilirubin which has been claimed to be the major interfering substance in the plasma does not contribute to the chromogen content of deproteinised plasma samples in the Jaffe reaction. Thus the claim that the acidification method for creatinine determination is non-specific as a result of bilirubin interfering with creatinine in the Jaffe reaction is without basis.

Another point that has also been raised against the specificity of the acidification method is that acidification not only destroys creatinine chromogen, but non-creatinine chromogen as well, the major one being bilirubin. This claim has not been substantiated by our study. A wide pH range of 4.5 to 12 had no significant effect on the colour due to bilirubin in solution, when reacted with alkaline picric acid. Thus in the direct colorimetric method described by Heinegard and Tiderstrom, adapted by Lolekha and Taksinamanee, acidification should have no significant effect on true creatinine levels, as bilirubin chromogen remains quite stable over this pH range. The claim by Lolekha and
Taksinamaneek that “the yellow pigment of bilirubin was partly destroyed by acid at pH 8.6 down to pH 5.8” was not substantiated by our study. Fading of the yellow colour of bilirubin resulting from reaction with alkaline picric acid, after acidification, was insignificant over the pH range 12 to 4.5. In our study bilirubin concentrations several times in excess (up to 200 μmol/l) of the normal, contributed very little to the total chromogen in the Jaffe reaction. Furthermore, even when the bilirubin concentration in solution was as high as 400 μmol/l, acidification caused insignificant fadding, (see fig. 3). We also failed to substantiate the claim by Lolekha and Taksinamaneek that bilirubin gave larger interfering values at pH 5 than at pH 7.8. Our results indicate very little or no difference in absorbance at these pH values when bilirubin solutions were reacted with alkaline picric acid (see fig. 3).

This study wishes to draw attention to the reliability of the simple but inexpensive method of acidification in the determination of plasma creatinine, while discounting claims levelled against this method questioning its accuracy on the basis of interfering substances particularly, bilirubin. This method has obvious advantages over more complex methods that are not only time-consuming and expensive but impractical in routine practice. In a group of Ghanaians studied, the plasma creatinine concentration measured by our method ranged between 44 and 78 μmol/l for females (mean 64.1 μmol/l; n = 60) and 47-106 μmol/l for males (mean 73 μmol/l; n = 44). These values compare favourably with values reported by Colombo et al using the Greiner Selective Analyser (GSA II), who also report good correlation between results obtained by their method and more standard methods based on the Jaffe reaction.

It is also clear from this study that using our method, ingested drugs listed earlier in this paper are unlikely to be major sources of error in the estimation of plasma creatinine levels. Such drugs may become important interfering substances when their blood levels exceed therapeutic levels.

The high content of non-creatinine chromogens in the plasma of normal Ghanaians (24-62% of normal creatinine concentration) must be borne in mind when the plasma creatinine levels are measured, in the investigation of renal diseases. The plasma creatinine concentration could be fortuitously high due to the high levels of plasma non-creatinine chromogens in such individuals.

Finally the nature of non-creatinine chromogens in the plasma of normal Ghanaians needs to be investigated further, since their plasma levels exceed by far the amounts reported by other workers, and since bilirubin is not a major interfering substance in the Jaffe reaction, using the modified method reported here.

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References


