Analyses on Heat-coagulated Blood and Serum

The Determination of Creatinine


The "standard" heat-clot method of London and Marymont is shown here to be applicable to the determination of creatinine. With this method creatinine can be assayed in very small quantities of serum, and only a conventional laboratory reagent, alkaline picrate, is required. A new fast technic has been developed in which extraction of the clot is carried out for only 10 min. at a high temperature. In addition, the heat-clot method used in conjunction with Lloyd's reagent lends itself to the determination of "true" creatinine values.

Recent papers (1-4) have outlined some uses of a heat-clot deproteination extraction procedure; in this paper it is applied to the measurement of serum creatinine. In contrast with the widely used method of Haden (5), in which there may be adsorption of creatinine onto the tungstic acid protein precipitate, here recovery is complete. Other methods of freeing creatinine of protein require considerable time and a fairly large volume of serum. The method of rapid extraction described here is probably applicable to a variety of serum constituents. The ability to obtain rapid analysis gives this test procedure a great advantage over other manual methods.

Materials and Methods

Reagents

Stock creatinine standard 150 mg./100 ml. in 0.1 N HCl. Store in the refrigerator.

Dilute creatinine standards Prepare 2, 4, 6, 9, 12, and 15 mg./100 ml. solutions by appropriate dilutions of the stock standard with water just prior to use. These solutions are diluted in the same manner as the serum, 1 part sample and 10 parts water (see Procedures).

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Saturated picric acid Dissolve 12 gm. of picric acid in 1 L. of demineralized water at 37° and cool to room temperature.

Sodium hydroxide, 2.5 N

Alkaline picrate reagent I Prepare before using by addition of 2 ml. of 2.5 N NaOH to 10 ml. of saturated picric acid.

Lloyd's reagent slurry Harleeo Lloyd's reagent (Hartman-Leeddon Co.) 600 mg./100 ml. in 0.1 N H₂SO₄.

Alkaline picrate reagent II Prepare before using by addition of 4.0 ml. of 2.5 N NaOH and 48.0 ml. of water to 20.0 ml. of saturated picric acid.

Procedures

Standard Heat-Clot Method

Place 0.2 ml. of serum in a flat bottom 25- × 95-mm. shell vial, cover tightly with aluminum foil to minimize evaporation, and immerse in boiling water for 2 min. Add 2.0 ml. of water, re-cover, and place in a 37° water bath for 60 min. To a 1.5-ml. aliquot of extract, add 0.75 ml. of alkaline picrate reagent I, substituting 1.5 ml. of water for blank and 1.5 ml. of creatinine solution (see below) for standard, incubate at room temperature for exactly 15 min., and measure absorbance in a Klett-Summerson colorimeter with a No. 50 filter. The average colorimeter reading corresponding to 2.0 mg./100 ml. creatinine is 45 Klett units.

After the heat-clot and subsequent extraction operations, water condensed on the walls of the vials is washed down with the extract itself. This must be done to achieve the greatest accuracy.

Color development is not a linear function of the concentration (6,7), and therefore a calibration curve is prepared by adding 0.2 ml. of dilute creatinine standards to 2.0 ml. of water, treating 1.5 ml-aliquots the same as extracts and graphing absorbance against concentration. The serum creatinine values are obtained from this standard curve. The standard curve for this method is linear up to 6.0 mg./100 ml. creatinine. Therefore, any standard below this value can be used in calculating normal creatinine values.

Heat-Clot with Lloyd's Reagent

Heat-clots are prepared as already described, covered with 5.0 ml. of Lloyd's reagent slurry, and extracted 60 min. at 37° with mixing by gentle rotation at 5-min. intervals. The slurry is decanted, and the vials washed with an additional 1.0 ml. of 0.1 N H₂SO₄, which is added to the slurry. The mixture is centrifuged at 1500 g for 20 min. and the supernatant fluid discarded. Then 3.0 ml. of alkaline picrate II is added to the pellet. The tubes are agitated vigorously for a total of 10 min.
Table 1. Recovery of Creatinine by 3 Methods Compared

<table>
<thead>
<tr>
<th>Creatinine added (mg./100 ml.)</th>
<th>Experimental values</th>
<th>Theoretical values</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Std. heat-clot</td>
<td>Lloyd's heat-clot</td>
<td>Haden</td>
</tr>
<tr>
<td>0</td>
<td>1.21</td>
<td>1.40</td>
<td>1.00</td>
</tr>
<tr>
<td>3</td>
<td>4.18</td>
<td>4.30</td>
<td>3.70</td>
</tr>
<tr>
<td>6</td>
<td>7.04</td>
<td>6.80</td>
<td>6.40</td>
</tr>
<tr>
<td>9</td>
<td>10.02</td>
<td>9.80</td>
<td>9.10</td>
</tr>
<tr>
<td>12</td>
<td>13.3</td>
<td>12.7</td>
<td>11.9</td>
</tr>
<tr>
<td>15</td>
<td>16.7</td>
<td>16.2</td>
<td>15.1</td>
</tr>
</tbody>
</table>
and centrifuged as previously. The absorbance of the supernatant fluid is measured against a blank prepared by substitution of water for serum. A standard curve is prepared by substitution of dilute creatinine standards for serum, and serum creatinine values are obtained from it.

Ten-Minute Extraction Method

Heat-clot 0.2 ml. serum samples for 2 min. As soon as possible overlay with 2.0 ml. of water, washing down the sides of the vial. Recover and return to the still boiling water bath. Turn off the flame immediately and leave for 10 min. Remove vials and cool to room temperature. Wash down the walls as before with supernatant extract and develop color on 1.5-ml. aliquots as described. In our arrangement, the temperature usually dropped to 72° by the end of the 10-min. extraction period.

Results

Recovery studies shown in Table 1 were performed by addition of aqueous creatinine to pools of serum. Measurements were made by the standard heat-clot, Lloyd’s reagent with heat-clot, and the Haden method. Recoveries by the Haden technic are invariably low, supporting the contention of Roscoe (8) and Owen et al. (9) that under these conditions there is adsorption of creatinine to protein during precipitation. Recoveries by the standard heat-clot method were complete. Recoveries by the heat-clot method with Lloyd’s reagent are not quite complete, but did average over 95%.

The results of a comparison of serum creatinine values obtained by 10 min. extraction time in slowly cooling boiled water (97–72°) and by the standard heat-clot method (60 min. extraction time at 37°) shown in Fig. 1 for 16 serums demonstrate the validity of rapid extraction.

Using our standard heat-clot method, a series of 12 replicates was run on pooled normal serum. The average value was 1.22 mg./100 ml., and the standard deviation was 0.67 mg./100 ml.

The extraction rate is a function of temperature, and the times necessary for 100% extraction at 4 different temperatures are shown in Table 2. It also illustrates that no interference by protein breakdown

<table>
<thead>
<tr>
<th>Temp.</th>
<th>Min. time for 100% extr. (min.)</th>
<th>Max. extr. time tested (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80–90</td>
<td>8</td>
<td>30</td>
</tr>
<tr>
<td>60</td>
<td>16</td>
<td>60</td>
</tr>
<tr>
<td>37</td>
<td>30</td>
<td>120</td>
</tr>
<tr>
<td>25</td>
<td>45</td>
<td>180</td>
</tr>
</tbody>
</table>

* No clot breakdown during these lengths of time. These are not the full lengths of time of clot stability.
occurs even at the higher temperature for longer periods of time. The temperature of 80–90° was the highest chosen since it was known that extraction cannot be accomplished at 100° because of the breakdown of the clot after 4 min. and the resulting protein interference (2).

Discussion

The pH of the extract or protein free filtrate is important because, for a given creatinine concentration, the color intensity of alkaline picrate increases as pH decreases. The pH of serum filtrates will vary with protein concentration to a much greater extent than extracts prepared with a neutral solution, and this contributes to the accuracy of our procedure.

![Graph showing comparison of creatinine values](image)

Fig. 1. Comparison of creatinine values (mg./100 ml.) obtained by heat-clotted serums extracted at different temperatures.

The heat-clot technic is well suited for use with very small volumes of serum. When extraction is performed for 10 min. at high temperature, the creatinine concentration in serum can be determined accurately, with great speed, and with minimum manipulative effort.

In this laboratory it was found that the 10-min. extraction technic as developed for creatinine could be applied in exactly the same fashion to the extraction of urate and NH+4 in the analyses of uric acid (2) and urea (1) by the heat-clot methods.
References