An Evaluation of Four Methods of Measuring Urinary Creatinine

James Martin Cooper and H. G. Biggs

Four different methods were evaluated for the determination of the creatinine content of a series of 24-hour urine samples. The results indicate that the methods of Folin, Hare, and Van Pilsum give essentially the same values. The precision of the method of Sullivan and Irreverre was extremely poor and the values obtained were 2-8 times higher than those obtained by the other technics.

The compound, 1,4-naphthoquinone, used in the procedure of Sullivan and Irreverre, was synthesized and compared with a commercial sample. Data concerning 1,4-naphthoquinone-2-potassium sulfonate is given to clarify its identity.

Numerous procedures have been devised for the measurement of creatinine, but unfortunately none of the methods is suitable for every application.

Among the chemical technics for the measurement of creatinine, the procedure of Van Pilsum (1) appears to be highly specific. This specificity is achieved through the degradation of creatinine to methylguanidine, a compound infrequently encountered in nature. The methylguanidine is estimated by the Sakaguchi color reaction (2), which is apparently limited to monosubstituted guanidines. The use of the Van Pilsum technic as a routine clinical chemistry procedure is contraindicated because of the numerous manipulations and reagents required. The method of Folin (3) has long been used as a routine procedure because of its speed and simplicity, even

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though it is generally accepted that this technic is not specific for creatinine. Hare (4) has increased the specificity of the Folin procedure through the use of Lloyd's reagent. The creatinine is separated from other constituents by absorption on the Lloyd's reagent. The creatinine is then eluted and the color developed by reaction with alkaline picrate.

Recently, Sullivan and Irreverre (5) published a colorimetric procedure for measuring creatinine which employs potassium-1,4-naphthoquinone-2-sulfonate (NQS). They presented data showing that the NQS method was specific for creatinine in that a number of compounds with structures similar to creatinine failed to yield a colored product with this reagent. Values were also given for urinary creatinine which were less than those obtained by the method of Folin (3). These lower values were attributed to the greater specificity of the NQS technic.

The investigation reported here is an evaluation of the procedures of Folin, Hare, and Sullivan and Irreverre as routine clinical chemistry methods. The method of Van Pilsum, because of its specificity, was used as the reference procedure.

**Materials and Methods**

The urine samples used in this study were carefully collected 24-hour specimens, obtained from apparently healthy individuals undergoing metabolic studies. Tests for reducing substances, protein, and ketone bodies were not performed on all the urine samples, but frequent urinalyses on specimens from these individuals never revealed the presence of these substances. The effects of these substances on the creatinine results were not evaluated in this study.

**Method of Folin**

The procedure used in this study was a modification of the Folin method (3), details of which are described in another report (6). The protocol was as follows: 1.0-ml. samples of urine or standard creatinine solutions were placed in 100-ml. volumetric flasks. Then 10 ml. of saturated picric acid was added, followed by 1.5 ml. of 10% NaOH. The contents of the flasks were thoroughly mixed and allowed to stand for 10 min. before dilution to volume. After the contents were thoroughly mixed again, the absorbances of the colored solutions were measured with a Klett-Summerson colorimeter using the No. 54
filter. Reagent blanks were prepared in the same manner except that water replaced the test solution.

Procedure of Hare

The original method of Hare (4) was modified to eliminate certain steps so that the entire procedure can be performed in matched colorimeter tubes. Also, more concentrated reagents were used to yield greater color intensities, in order that the absorbances could be measured with a Coleman Jr. spectrophotometer.

Materials

The materials employed were those described by Hare (4) except for the following:

Alkaline picrate solution. This was prepared by mixing 5 volumes of 0.04N picric acid and 1 volume of 10% NaOH. This reagent is unstable and must be used promptly after preparation.

Lloyd's reagent. The fine particles of this material which did not sediment readily in distilled water were removed by decanting after centrifuging. The reagent was suspended twice in acetone and then several times in water until the supernatants were clear.

Standard solutions. These were prepared by diluting the proper volumes of a stock solution of 1 mg./ml. of creatinine in 0.1N HCl to 100 ml. with water. Standards were prepared containing 0.005, 0.01, 0.02, 0.04, and 0.08 mg./ml. of creatinine.

Technics

Two-ml. samples of urine, previously diluted 1:100, were added to matched colorimeter tubes. Standards were prepared by adding 2.0 ml. of the above-mentioned standard solutions (0.005 and 0.02 mg./ml. were used in routine determinations) to similar tubes. Reagent blanks were prepared using 2.0 ml. of distilled water. To each tube was added 0.20 ml. of saturated solution of oxalic acid and 10-20 mg. (approximated) of Lloyd’s reagent. After a 10-min. period of frequent shaking, the tubes were centrifuged and the supernatants were discarded. Three milliliters of the alkaline picrate solution were added to the precipitate in each tube and allowed to stand 10 min. with occasional shaking. The tubes were then centrifuged and the absorbancies of the supernatants measured in a Coleman Jr. spectrophotometer at 520 mμ without decanting.

The absorbance-concentration relationship for this method is not
a linear function, but because the values obtained for known amounts of creatinine were so constant, it was possible to prepare a calibration curve. Values for this curve were obtained by using 0.005-, 0.01-, 0.02-, 0.04-, and 0.08-mg. portions of creatinine. The use of standard creatinine solutions during the routine urine determinations served only as controls.

**Method of Van Pilsum**

The reagents and procedure as described by Van Pilsum (1) were used with two changes. One change was the use of the urine specimen diluted 100 times rather than the 200-fold dilution prescribed in the original procedure. The other change was in the step where creatinine is converted to methylguanidine. This reaction was carried out in a boiling-water bath, rather than at room temperature, to obtain better reproducibility and greater color development.

The absorbance values, as measured with a Coleman Jr. spectrophotometer at 515 m\(\mu\), for known concentrations of creatinine do not describe a straight line when plotted against concentration. Therefore, it was necessary to prepare a calibration curve. This was accomplished by the use of known amounts of creatinine from 10-250 \(\mu\)g. per sample. The reproducibility of the results was excellent when the reactions during the color development were accurately timed. Standard solutions of creatinine were used as controls during the measurement of urinary creatinine.

**Procedure of Sullivan and Irreverre**

This procedure (5) was altered by reducing all of the volumes to one-half of the original. A reagent blank was prepared by substituting 1.0 ml of 0.1N HCl for the sample. The colored products were extracted into 3.0-ml portions of methylene chloride and the absorbancies were measured in a Coleman Jr. spectrophotometer at 540 m\(\mu\) without removing the aqueous layers.

It was also impossible to prepare a calibration curve for this procedure, because, in spite of the fact that the absorbance-concentration relationship appears to be linear, the results were very inconsistent. Thus, it was necessary to use triplicate standard creatinine solutions, containing 0.1 and 0.3 mg. of creatinine, each time an analysis was performed. The average of the two most comparable values for each concentration of standards was used for calculation of the unknown creatinine values.
Results

Method of Folin

The results obtained utilizing this procedure show excellent precision. Duplicate determinations yield absorbancies within a range of ± 5 Klett units. The absorbance-concentration relationship is not a linear function. However, the excellent reproducibility of standards allows the utilization of a calibration curve.

Procedure of Hare

Although the concentration of creatinine measured by this method was increased fivefold from the original method, experimentation showed that it was necessary to increase the Lloyd’s reagent only twofold. This was fortunate, because the volume of Lloyd’s reagent, after centrifuging, was small enough so that absorbance measurements could be made on the supernatants without transfer to other tubes.

The absorbance measurements were made at 520 m\(\mu\), rather than 500 m\(\mu\) as originally specified, because of the smaller absorption of the reagent blanks at the longer wave length.

Procedure of Van Pilsum

It was observed that when a given amount of creatinine was degraded to methylguanidine by o-nitrobenzaldehyde at room temperature, according to the original procedure (1), inconsistent results were obtained. It was further observed that heating the tubes during this step yielded greater color intensities and also the results were much more consistent. The optimum heating time for this reaction was established at 20 min. by measuring the color developed after heating for various lengths of time. The results obtained when 100-\(\mu\)g. samples of creatinine were used are as follows.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Min.</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room temperature</td>
<td>20</td>
<td>0.083</td>
</tr>
<tr>
<td>Boiling water bath</td>
<td>10</td>
<td>0.247</td>
</tr>
<tr>
<td>Boiling water bath</td>
<td>20</td>
<td>0.253</td>
</tr>
<tr>
<td>Boiling water bath</td>
<td>30</td>
<td>0.235</td>
</tr>
<tr>
<td>Boiling water bath</td>
<td>40</td>
<td>0.187</td>
</tr>
</tbody>
</table>

Procedure of Sullivan and Irreverre

It was necessary to synthesize the NQS used in this study, because a sample of this material, obtained from the only commercial source (Borden Chemical Co.) was apparently impure, as it was dark brown.
and not as described in the literature. The compound, as the mono-
hydrate, was prepared according to the synthesis of Fieser and
Fieser (7). The crude product obtained by this procedure was puri-
fied by recrystallizing 3 times from water. The yield of final product
was approximately 50 per cent.

A question concerning the purity and identity of this compound
arose, because of the unexpected results obtained when it was used
in the creatinine analysis. A search of the literature failed to reveal
any distinguishing physical constants for this compound.

The derivative, 2-bis-1,4-naphthoquinone-2,2-diethyl malonate was
prepared according to the procedure of Pratt and Boehme (8). The
color changes during the reaction were those described by the au-
thors. The dark-blue product melted at 263-266°—literature values:
264.5-267° (8). The synthesized compound was analyzed for sulfur
by the method of Katz (9). The average result for duplicate deter-
minations was 10.87 per cent sulfur (theoretical: 10.88 per cent).

The commercial NQS, through treatment with charcoal and by re-
peated recrystallization, was obtained in a form that closely re-
sembled the material obtained by synthesis. Potassium analyses
were performed on samples of both, using a Patwin flame photome-
ter* according to the directions of the manufacturer. The values ob-
tained indicate that the synthesized compound contained 11.7 per
cent potassium and the commercial compound contained 11.2 per cent
potassium. The theoretical value is 13.29 per cent potassium.

The average results for the synthesized NQS show that it contains
only 88 per cent of the calculated amount of potassium. The results
for the commercial product show a value of 84.5 per cent of the
theoretical potassium content. In view of the fact that the sulfur
analysis gave essentially theoretical results, it appears probable that
the reason for the lower potassium values is that the salt underwent
hydrolysis during recrystallization.

To compare the synthesized and commercial compounds further,
the ultraviolet absorption spectra were examined employing a Beck-
man DU spectrophotometer. The two materials yielded essentially
identical results. Samples of both showed major absorption peaks at
250 and 254 mμ and minor peaks at 340, 380, and 400 mμ. The ab-
sorptions were affected by changes in pH, and, to avoid this varia-
tion, the extinction coefficients were measured in 0.06 M phosphate

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*Patwin Instruments, Waterbury, Conn
Results given in mg./ml. buffer at pH 7.15. The molar extinction coefficients (E<sub>254 cm<sup>-1</sub></sup>) for the two samples were: synthesized, 1.84 × 10<sup>4</sup>; commercial, 1.88 × 10<sup>4</sup>.

Infrared absorption spectra were made on the synthesized and commercial NQS, in potassium bromide pellets. The curves were essentially identical and they were typical of an aromatic sulfonate.

Several efforts were made to improve the results of the creatinine analyses. Various solvents were employed including ethylene chloride, carbon tetrachloride, chloroform, chlorobenzene, and methylene chloride. Ethylene chloride and methylene chloride were about equally effective and superior to the other solvents. Contamination of the glassware was ruled out by thorough washing in plastic containers with several detergents, followed by thorough rinsing with distilled water. To eliminate the possible effects of heavy metals, the glassware was washed with disodium ethylenediaminetetraacetate.

An investigation of reaction temperature was made in an attempt to improve the results. It was found that at low temperature (0°) the color produced gave extremely poor reproducibility, and was a blue-gray rather than the usual scarlet. At high temperatures, the results seemed to be approximately the same as those obtained when the reaction was conducted at room temperature.

Since the various manipulations did not seem to improve the reproducibility of the NQS method, further attempts to modify the procedure were abandoned.

Results and Discussion

A statistical analysis of representative results of the creatinine analyses is shown in Table 1. As can be seen in the table, the differ-

<table>
<thead>
<tr>
<th>Method*</th>
<th>Mean S.D.</th>
<th>Mean S.D.</th>
<th>Mean S.D.</th>
<th>Mean S.D.</th>
<th>95% Confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sullivan &amp; Hare</td>
<td>1.77 0.239</td>
<td>1.84 0.253</td>
<td>1.72 0.305</td>
<td>5.45 2.27</td>
<td>±1.20</td>
</tr>
<tr>
<td>Van Pilmen</td>
<td>1.24 0.151</td>
<td>1.29 0.111</td>
<td>0.99 0.087</td>
<td>3.74 2.37</td>
<td>±2.02</td>
</tr>
<tr>
<td>Folin</td>
<td>1.03 0.222</td>
<td>0.99 0.218</td>
<td>1.11 0.251</td>
<td>5.18 2.70</td>
<td>±2.3</td>
</tr>
<tr>
<td>Hare</td>
<td>0.85 0.149</td>
<td>.........</td>
<td>.........</td>
<td>6.41 2.79</td>
<td>±1.7</td>
</tr>
<tr>
<td>Plleum</td>
<td>0.92 0.278</td>
<td>.........</td>
<td>.........</td>
<td>5.30 2.41</td>
<td>±1.4</td>
</tr>
<tr>
<td>Sullivan &amp; Irrev</td>
<td>1.03 0.230</td>
<td>.........</td>
<td>.........</td>
<td>2.59 1.55</td>
<td>±1.02</td>
</tr>
</tbody>
</table>

*Results given in mg./ml.
ences in the means, for the methods of Folin, Hare, and Van Pilsum, in no case exceed the 95 per cent confidence limits. Contrary to this, when the means for the method of Sullivan and Irreverre are similarly compared with each of the other means, the differences invariably exceed the confidence limits.

The results shown in Table 1, also indicate that the more involved method of Hare offers no advantages over the procedure of Folin. These findings are in agreement with the report of Edwards and Whyte (11). These results also reveal that the method of Van Pilsum gave values which were not statistically different from the results of the other technics. This was unexpected because of the greater inherent specificity of the two-step degradative reaction. The close agreement of values of the method of Folin and Van Pilsum may be due in part to measurement of some creatine in addition to the creatinine. A recent report by Rauenbusch et al. (12) suggests that a reaction temperature of 70° or above leads to the degradation of creatine. This finding is in agreement with that of Martinez and Doolan (13).

Because the results obtained here with the Van Pilsum method are so similar to those obtained with the Folin procedure, there seems to be little advantage in using the more complex one.

As mentioned above, the procedure of Sullivan and Irreverre provides very poor reproducibility. An example of this is shown below

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 mg. Creatinine</td>
<td>.443</td>
</tr>
<tr>
<td>0.3 mg. Creatinine</td>
<td>.581</td>
</tr>
<tr>
<td>0.3 mg. Creatinine</td>
<td>.495</td>
</tr>
<tr>
<td>Urine A</td>
<td>.684</td>
</tr>
<tr>
<td>Urine A</td>
<td>.555</td>
</tr>
<tr>
<td>Urine A</td>
<td>.562</td>
</tr>
<tr>
<td>Urine B</td>
<td>.584</td>
</tr>
<tr>
<td>Urine B</td>
<td>.737</td>
</tr>
<tr>
<td>Urine B</td>
<td>.792</td>
</tr>
<tr>
<td>0.1 mg. Creatinine</td>
<td>.221</td>
</tr>
<tr>
<td>0.1 mg. Creatinine</td>
<td>.191</td>
</tr>
<tr>
<td>0.1 mg. Creatinine</td>
<td>.196</td>
</tr>
</tbody>
</table>

by the values obtained in multiple determinations using NQS. The results seen in Table 1 show that the procedure of Sullivan and Irreverre also has another defect, in that the creatinine values obtained using this technic are approximately 2-8 times greater than the values obtained by the other methods. Also, the variations with this method have no consistent relationship with the results of the
other procedures. The characterization of the synthesized and commercial NQS leaves little doubt concerning their correct identity; thus it seems that the difficulties experienced with the Sullivan and Irreverre technic (5) are not due to this material.

Conclusions

The results obtained in this investigation indicate that the procedures of Folin (3), Hare (4), and Van Pilsum (1) are all suitable for the routine measurement of urinary creatinine. However, the simplicity and the excellent reliability of the Folin procedure make it the method of choice.

References

2. Sakaguchi, S., J. Biochem. 5, 133 (1925).
10. Scheffe, I. H., Biometrika 40, 87 (1953).