A Modified Semi-automated Resorcinol Method for the Determination of Inulin

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A semi-automated resorcinol method for the determination of inulin in plasma and urine specimens, using standard AutoAnalyzer modules, is described. Plasma and other protein-containing fluids undergo a preliminary manual protein precipitation procedure. The deproteinized specimens are processed at a rate of 50/hr, with a high degree of accuracy and precision. The superiority of the present method is largely due to the elimination of small acid-resistant pump tubes from the manifold. This is possible because reagents are premixed prior to aspiration into the system, allowing simplification of manifold design over other automated methods.

THE RENAL CLEARANCE of inulin is generally considered to be the most accurate estimate of the renal glomerular filtration rate in many species. However, the difficulties involved in measuring the inulin clearance include the necessity of maintaining an infusion at a constant rate in order to replace urinary losses during the period of specimen collection, and the tedious and sometimes erratic analytic procedures used for the estimation of inulin concentrations in urine and plasma. This report sets forth a method by which the latter objection may be overcome. It is based on modifications of the manual methods of Roe, Epstein, and Goldstein, and of Higashi and Peters; and depends on the generation of a pink chromagen upon reaction of resorcinol with fructose, the acid hydrolysis product of inulin (1, 2). The simplicity of the present method derives from an observation by Smith, that reagents for the manual resorcinol procedure may be premixed and then added to suitably diluted biological fluid samples containing inulin (3).

Materials and Methods

Equipment

Standard AutoAnalyzer† modules are utilized. These include a sampling device, a proportioning pump, a heating bath at 80° con-

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taining an 80-ft time delay coil, and a colorimeter with 520-nm interference filters. A linearized recorder, giving deflections proportional to absorbance, is used in this laboratory.

The manifold is constructed as shown in Fig 1. Diluted protein-free samples, containing inulin at concentrations up to 4 mg/100 ml, are mixed with air-segmented stream containing an HCl-resorcinol reagent described below. After a color development period of approximately 10 min at 80°, the resulting chromagen passes through a colorimeter flow cell and the absorbance at 520 nm is measured. Acidflex tubing and connections are used for all lines containing the HCl-resorcinol reagent.

Reagents

Deproteinizing reagents Prepared as originally described by Somogyi (4). These include a 10% (w/v) solution of ZnSO₄·7H₂O and a 0.5 N NaOH solution. Concentrations are adjusted so that 11 ml of the NaOH solution neutralizes 10 ml of the ZnSO₄ to pH 9.

Stock resorcinol-thiourea reagent Dissolve 2.0 g resorcinol and 5.0 g thiourea to a total volume of 1000 ml in glacial acetic acid with
gentle heating. This reagent may be stored for several weeks at room
temperature in an amber bottle.

Inulin standard solutions  Prepared by diluting a stock solution
of purified inulin containing 100 mg/100 ml. Inulin concentrations in
working standards range from 0.1 to 4.0 mg/100 ml. These solutions
are stable for at least 2 weeks at 4°C.

Working HCl-resorcinol reagent  Add 20 ml of stock resorcinol-
thiourea solution to 1000 ml conc. HCl. To this, add 7.5 mg FeCl₃·6H₂O
and mix. This solution is aspirated directly into the AutoAnalyzer
(Fig 1). It is made up on the day of use and is stable for at least 6 hr
at room temperature.

Preparation of Samples and Analytic Procedure

Plasma and other protein-containing specimens are deproteinized
by adding 0.5 ml of sample to 7.5 ml H₂O, followed by the successive
addition of 1.0 ml 10% ZnSO₄·7H₂O and 1.0 ml 0.5-N NaOH, with
mixing after each. After centrifuging at 3500 rpm for 15 min the
 supernatant is decanted into an AutoAnalyzer cup. This protein-
 precipitation procedure gives a 1:20 dilution of the original sample,
and is thereby suitable for specimens containing inulin in concentra-
tions up to 80 mg/100 ml without further dilution. Normal urine and
other protein-free fluids are diluted so that the final inulin concentra-
tions are less than 4 mg/100 ml.

After the establishment of a stable baseline, suitably diluted and
deproteinized specimens are aspirated into the manifold at a rate of
50/hr, using a 2:1 sample-to-wash ratio. After peaks from all samples
have been recorded, the reagent line and the sampling tube are al-
lowed to aspirate air until the coils and tubing are empty. Washing
with water tends to cause erratic performance during subsequent
usage of the system and necessitates a prolonged period of reagent
aspiration before baseline stabilization can be re-established.

Results

Recoveries of inulin added to plasma and urine are essentially com-
plete (Table 1 and Fig 2). Mean recoveries and recovery ranges are
similar to values previously reported for manual methods. Plasma
recovery experiments with added inulin at 20 and 40 mg/100 ml, per-
formed on different days, were uniformly satisfactory (Table 1). Also,
the recovery of inulin from plasma and urine specimens, over a wide
range of concentrations, was complete (Fig 2). In this group, plasma
recoveries ranged from 99 to 103% (mean, 101 ± 2% SD), while urine
recoveries ranged from 98 to 105% (mean, 102 ± 3% SD). Plasma inulin
Table 1. Recoveries of Inulin Added to Plasma (mg/100 ml)

<table>
<thead>
<tr>
<th>Amount added</th>
<th>No. of specimens</th>
<th>Mean recovery</th>
<th>Range</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>20</td>
<td>19.98</td>
<td>19.4–20.7</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(99.9)</td>
<td>(97.0–103.5)</td>
<td>(1.5)</td>
</tr>
<tr>
<td>40</td>
<td>19</td>
<td>39.84</td>
<td>38.1–41.7</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(99.6)</td>
<td>(95.3–104.3)</td>
<td>(2.1)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.

Fig 2. Recovery of added inulin from plasma and urine by modified semi-automated resorcinol procedure. Recoveries of added inulin from plasma samples, in amounts ranging from 10 to 100 mg/100 ml, varied from 99 to 103% (mean, 101 ± 2%, SD). In urine, recoveries of amounts from 25 to 250 mg/100 ml ranged from 98 to 105% (mean, 102 ± 3%, SD).

Concentrations must be corrected by subtracting an “inulinoid blank,” measured on a plasma specimen obtained prior to the administration of inulin. In normal subjects, the subtraction of a urinary inulinoid blank is unnecessary.

Duplicate determinations agreed closely (Fig 3). Chromagen absorbance at 520 nm is a nearly linear function of inulin concentration, and interaction between specimens is minimal. When the same specimen was repeatedly sampled, inulin concentration values agreed within 0.02 mg/100 ml. The manual protein precipitation procedure for plasma does not impair analytic precision significantly. In 19 pairs of duplicate precipitations of plasma specimens containing added inulin at concentrations of 20 and 40 mg/100 ml, the standard deviation between duplicates was ±0.24 mg/100 ml.

Discussion

This semi-automated method has proved reliable and precise during extensive use in this laboratory for the analysis of inulin in renal...
clearance specimens. In common with most other methods, plasma samples must be deproteinized manually (5, 6). However, if an automatic dilutor is used for the initial dilution of the sample with water and automatic pipets are used for the addition of the protein-precipitating reagents, this step presents no great difficulty. Although a

Fig 3. Unretouched photograph of recorder tracing of chroomagen absorbance from inulin standards at concentrations of 0.1, 0.5, 1.0, 2.0, 3.0, and 4.0 mg/100 ml, arranged in ascending and descending order. Note lack of interaction between adjacent specimens. Sampling rate, 50/hr; sample-to-wash ratio, 2:1; chart speed, 10 in./hr.
completely automated method depending upon the dialysis of the fructose hydrolysis product of inulin across an AutoAnalyzer dialyzer membrane has been proposed, it suffers from the necessity of a slow rate of sample aspiration as well as a theoretical possibility that errors due to changes in circulating plasma fructose levels will be maximized (7).

The premixing of hydrochloric acid and the resorcinol reagent is an innovation of the present method. Semi-automated resorcinol methods for inulin, reported previously, have required the pumping of a resorcinol reagent through a separate small Acidflex pump tube to a location on the manifold where the reagent is combined with a hydrochloric acid dilution of the sample. In the author’s experience, Acidflex pump tubes of small size often perform erratically and result in poor reproducibility. The manifold described herein uses Acidflex pump tubes of medium diameter; 2 such tubes are hooked in parallel for the aspiration of the hydrochloric acid-resorcinol reagent and an additional pair pull solution through the colorimeter. The reproducibility of chromagen development is excellent, and cross-contamination of samples is minimal when specimens are processed at a rate of 50/hr (Fig 3). Absorbance is nearly linear for aspirated samples containing inulin in concentrations up to 4 mg/100 ml.

The addition of ferric chloride, as suggested by Higashi and Peters, appears to enhance the stability and reproducibility of color development (3). Although they suggested that maximum chromagen absorbance occurred at 480 nm, the maximum absorbance relative to baseline for the present semi-automated method occurs between 505 and 520 nm, using standard AutoAnalyzer filters. While absorbance is essentially constant over this range, it exceeds that at 480 nm by 12%.

Although the reagents are premixed in the present method, the hydrolysis of inulin to fructose is complete. On a weight basis, samples of fructose result in chromagen development equivalent to the same amount of inulin. If glucose-containing samples are aspirated, absorbance also occurs linearly with glucose concentration. However, the sensitivity of the method, by weight, for inulin is 27 times that for glucose. Since the “inulinoid blanks” in plasma from normal fasting subjects range from 2 to 3 mg/100 ml, this amount of noninulin chromagen may be related to normal fasting plasma glucose concentrations.

References