A Method For Quantitative Extraction of Urinary 5-Hydroxyindoleacetic Acid

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A method for the quantitative assay of urinary 5-hydroxyindoleacetic acid (5 HI-AAA) is presented. The limits of accuracy and precision obtained reflect good recovery and reproducibility. The relatively high recovery is largely due to maintaining a low distribution coefficient of 5-HIAA during purification of the aqueous extract with chloroform.

The quantitative estimation of urinary 5-hydroxyindoleacetic acid (5-HIAA) has attained clinical importance with increasing interest in indole metabolism. Urinary 5-HIAA levels have largely been determined by the use of an extraction procedure described by Udenfriend et al. (1). The author has found, as have others (2), that this method produces inconsistent results on varying dilutions of a given urine sample and suggests the following extraction procedure to circumvent the source of error.

Materials and Methods

1. Ethyl ether  Anhydrous, reagent grade
2. Chloroform  Reagent grade
3. Sodium chloride  Reagent grade
4. Sodium hydroxide, 1.0 N
5. Hydrochloric acid, 2.0 N
6. Udenfriend’s reagents  100 mg. nitrosonaphthol in 100 ml. ethanol, and freshly prepared nitrous acid according to directions (1).

The Beckman Model DU spectrophotometer and cuvets or the Bausch and Lomb Spectronic 20 and cuvets is used.

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Procedure

Add 10 ml. urine to a glass-stoppered centrifuge tube containing approximately 3 gm. NaCl, and adjust the pH to 7 with 1.0N NaOH. Shake the urine for 1 min. and then centrifuge at 1800 rpm for 10 min. Transfer the supernatant liquid to a 60-ml. glass-stoppered centrifuge tube, and wash the residue with about 5 ml. saturated NaCl solution. Combine the washings after centrifugation with the urine supernate and discard the precipitate.

Adjust the purified urine to pH 2 with 2.0N HCl, combine with excess NaCl, and extract twice with 20 ml. ethyl ether, shaking each time for 3 min. After centrifugation, aspirate the ether solution, transfer to an evaporating dish, and evaporate to dryness. Resuspend the dry extract in 4 ml. distilled water, transfer to a test tube containing 10 ml. chloroform, and shake vigorously for about 3 min. After centrifugation, transfer 3 ml. of the aqueous layer to a clean test tube to which then add 1.5 ml. nitrosonaphthol solution and 1.5 ml. nitrous acid (1). After 5 min. extract the mixture with ethyl acetate to remove unreacted reagents and other contaminants. Transfer a portion of the aqueous layer to a cuvet for measurement of absorbance at 540 m\mu, and determine the concentration by extrapolation from a standard curve.

Table 1. Recovery of 5-Hydroxyindoleacetic Acid

<table>
<thead>
<tr>
<th>Amt. of sample (ml.)</th>
<th>Added</th>
<th>HIAA (sp.) Recovered*</th>
<th>% Recovered</th>
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*Calculated as per cent recovery of total 5-HIAA added.
†Aliquots of a single specimen.
Results

Limits of reproducibility and recovery inherent in this procedure were determined from urine and aqueous solutions of 5-HIAA. Results obtained from 20 replicate urine samples averaged 36 µg. (S.D. ± 1.7 µg.). Twenty 10-ml aliquots of aqueous solution containing 40 µg 5-HIAA/10 ml. yielded a mean value of 39 µg. (S.D. ± 1.7), reflecting as do the replicate urine results, a high level of reproducibility.

Recovery of 5-HIAA from standard urinary and aqueous solutions of varying concentrations ranged from 90 to 105%, calculated as recovery of total 5-HIAA added (Table 1). These data compare favorably with the results reported by Udenfriend et al. (1), from which the recovery of total 5-HIAA added can be calculated as 60-93%.

Discussion

The extraction procedure is summarized as: (1) preliminary removal of inorganic and organic solutes, (2) ether extraction of 5-HIAA with resuspension of the subsequently dried extract in distilled water, and (3) purification of the aqueous extract with chloroform.

The 5-HIAA and other substances with low distribution coefficients are extracted from acid, salt-saturated urine into neutral polar and nonpolar organic solvents. Because false spectrophotometric readings may occur in the presence of normal urinary constituents other than 5-HIAA (3), organic and inorganic urinary solutes are first precipitated from neutralized, salt-saturated urine prior to extraction of 5-HIAA. Removal of the precipitate considerably diminishes the volume of the salt-saturated urine sample, and to avoid volumetric inaccuracies, the entire sample rather than an aliquot thereof is extracted with ether.

Care must be taken when neutralizing the urine to avoid raising the pH above 7, as 5-HIAA is progressively destroyed in basic solutions, with consequent poor recovery. Destruction of 5-HIAA also occurs on prolonged exposure to air at room temperature, requiring that the ether extract be resuspended in water immediately upon drying.

The aqueous solution of 5-HIAA is then further purified by extraction of contaminants with chloroform. Chloroform purification of acidified urine prior to ether extraction (1) is better avoided, as seen from the following.

Forty microliters of solution containing 1 µg 5-HIAA per microliter H₂O were added to each of 12 tubes containing, in consecutive

Twenty milliliters chloroform were added to the nine tubes containing acidified saline solution. After the tubes were shaken for a few minutes and centrifuged, the chloroform was removed and replaced with 20 ml. fresh chloroform. The tubes were again shaken and centrifuged. Then, 3 ml. of the aqueous supernates were removed and reacted with Udenfriend’s reagent. After purification with ethyl acetate, the absorbance of the solutions was read at 540 mμ.

An average of determinations in each series was taken and calculated as percentage of the control average.

Fig. 1 illustrates losses of up to 50% of 5-HIAA from acidified solutions of NaCl within the normal range of urine milliosmolarity (4). The amount of 5-HIAA inevitably lost into chloroform from a given urine sample is proportional to the total solute concentration and to the acidity of solution, varying with these factors. Avoidance of the salting-out of 5-HIAA into chloroform largely accounts for the relatively high recovery and reproducibility obtained with this method.

*1000 mosm. equals 32.35 gm. NaCl/kg. H₂O; 1200 mosm. equals 38.86 gm. NaCl/kg. H₂O; 1400 mosm. equals 45.22 gm. NaCl/kg. H₂O. Proper allowances were made for the activity coefficients of NaCl at the various concentrations (5).
References