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A RAPID QUANTITATIVE METHOD FOR THE ESTIMATION OF 5-HYDROXYINDOLEACETIC ACID IN HUMAN URINE

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Abstract—5-Hydroxyindoleacetic acid in urine adjusted to 0·1N with HCl, is selectively adsorbed on G-10 Sephadex. Interfering fluorophores are then leached out and the acid eluted with 0·02N NH_4OH, the eluate adjusted to 3N with concentrated HCl and estimated by spectrophotofluorimetry. The method is simple and quick, and its high degree of specificity and sensitivity enables it to be used on urine volumes of less than 1 ml. Recoveries of 90% and more have been obtained with 5-hydroxyindoleacetic acid added to urine.

AN INCREASED urinary excretion of 5-hydroxyindoleacetic acid (5HIAA), the principle metabolite of 5-hydroxytryptamine (5HT), has been established as a critical index of the carcinoid syndrome,[1, 2] and in the last few years, interest in the relationship of 5HT to diseases has been considerably increased. It is for this reason that the excretion of 5HIAA has come under close scrutiny and since publication of a method for its quantitative estimation in urine,[3] several authors have tried to modify the original method with a view to improving its sensitivity and specificity.[4–6] However, none of these methods were suitable for our requirements which needed a short, simple method capable of estimating 5HIAA with the highest possible sensitivity and specificity, in the very small volumes of urine present in 12–16-week-old foetuses.

A method has therefore been devised based on the observation,[7] that 5HIAA in urine adjusted to 0·1N with HCl was adsorbed on G-10 Sephadex and could be quantitatively eluted from it in a small volume of 0·02N NH_4OH. The 5HIAA was then estimated fluorimetrically by established techniques for 5-hydroxyindoles.[8]

METHODS

Preparation of column

G-10 Sephadex (1 g) was slurried in 0·1N HCl and poured into a glass column (internal diameter 1 cm, height 35 cm). The Sephadex was allowed to settle by gravity on a base formed by a pledget of cotton wool taking the usual precautions for prevention of air bubbles in the column.[9]

Treatment of urine

Column procedure. One millilitre of urine mixed with 10 ml of 0·1N HCl was allowed to drain through the Sephadex column which was then washed with 10 ml of 0·1N HCl, followed by 5 ml of distilled water. The adsorbed 5HIAA was eluted with 5 ml

of 0·02N NH_4OH, and a suitable aliquot (e.g. 1 ml) of this eluate, diluted with an equal volume of 6N HCl. The relative fluorescence intensity (RFI) of this solution was determined in an Aminco Bowman Spectrophotofluorimeter set for activation at 295 mμ and fluorescence at 535 mμ. Since the RFI of the solution was found to decrease with time after the addition of 6N HCl, the estimation was carried out within 15 min of the addition of the acid. One drop of a saturated solution of potassium persulphate in water was next added to the solution with thorough mixing. After 2–3 min, the RFI of the mixture was again determined, and this value which represented the urine "blank", was subtracted from the original RFI to give the corrected RFI of 5HIAA. If the 5HIAA concentration was high, it took as much as 5 min to reach the final "blank" value for the RFI after the addition of the potassium persulphate. If necessary, the decline in RFI could be followed by observing the needle on the photometer and reading the value when the needle was finally stationary.

Figure 1 shows the fluorescence spectrums of (a) adult male human urine and (b) pure 5HIAA, both of which had been adjusted to 3N with HCl. The activation wavelength was set at 295 mμ.

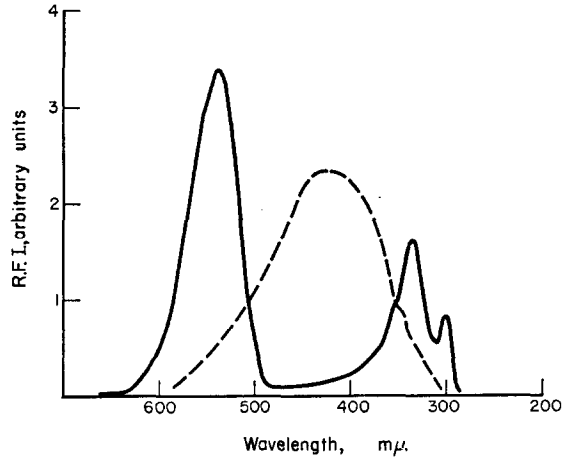

FIG. 1. Fluorescence spectrum of (a) urine (----) and (b) 5HIAA (——), both of which were adjusted to 3N with concentrated HCl, and activated at 295 mμ.

Figure 2 shows the fluorescence spectrums of the urinary 5HIAA obtained from the NH_4OH eluate off the column (a) before and (b) after, the addition of potassium persulphate.

The spectrophotofluorimeter was standardized by adjusting the RFI of a standard solution of quinine in 0·1N H_2SO_4 (activation wavelength 355 mμ, fluorescence wavelength 455 mμ) to a known value, by means of the sensitivity control. This setting was checked several times during the course of estimations, and necessary adjustments made to compensate for the decrease in intensity of the Xenon lamp with time.

The characteristics of the instrument were:

Slit arrangement—No. 4.

Lamp—XBO—150 W.

Detector—IP21 photomultiplier tube.

Batch procedure. One gram of Sephadex G-10 was added to a mixture of 1 ml of urine with 10 ml of 0·1N HCl in a centrifuge tube. The Sephadex was agitated for 30 sec in the mixture by means of a Vortex Junior mixer (Scientific Industries Inc., Queens Village, N.Y.) and packed by centrifugation. The supernatant solution was discarded and the Sephadex washed by repeating the process of agitation, centrifugation and discarding of the supernatant solutions after addition of 10 ml of 0·1N HCl, followed by 5 ml of distilled water. Finally, the supernatant solution obtained by following the above operations after addition of 5 ml of 0·02N NH_4OH, was treated in the same manner as the NH_4OH eluate obtained from the column.

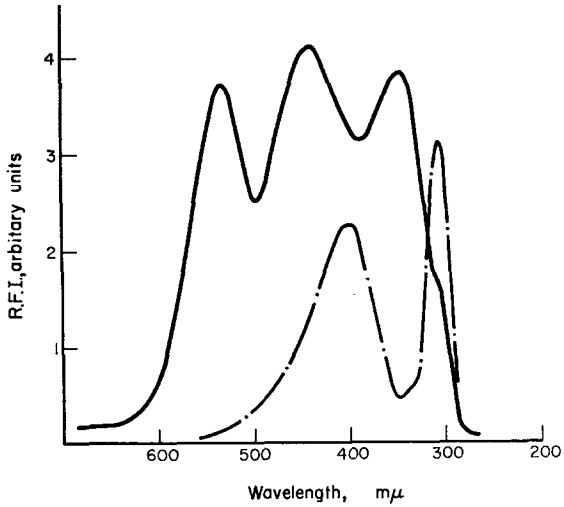

FIG. 2. Fluorescence spectrum of 0·02N NH_4OH eluate from G-10 Sephadex column, adjusted to 3N with concentrated HCl, activated at 295 mμ, before (——) and after (—•—•) the addition of potassium persulphate.

Standard curves for 5HIAA

5HIAA was dissolved (a) in water (b) 0·02N NH_4OH and diluted to give concentrations between 1 μg and 10 μg per ml. The solutions were adjusted to 3N with concentrated HCl and the RFI determined immediately. Corrected values of the RFI were obtained by subtracting the "blank" readings after the addition of the potassium persulphate solution, and the standard curves plotted.

RESULTS

Recovery of 5HIAA *added to urine by column procedure*

One millitre of a standard solution of 5HIAA was added to (a) 10 ml of 0·1N HCl (b) 1 ml of urine in 10 ml of 0·1N HCl. The two different mixtures were each treated by the column procedure. Table 1 shows the results of a typical recovery experiment.

The average recovery of added 5HIAA from four different experiments was 94 ± 2 per cent.

Recovery of 5HIAA *added to urine by batch procedure*

One millitre of a standard solution of 5HIAA was added to (a) 10 ml of 0·1N HCl (b) 1 ml of urine + 10 ml of 0·1N HCl. The two different mixtures were each treated

by the batch procedure. Table 2 shows the results of a typical recovery experiment.

The average recovery of added 5HIAA from five different experiments was 82 ± 5 per cent.

Stability of 5HIAA *in* 0·02N NH_4OH

The standard curve obtained for 5HIAA dissolved in water was identical with the standard curve obtained after allowing 5HIAA dissolved in 0·02N NH_4OH to stand for 3 hr at room temperature.

TABLE 1. RECOVERIES OF 5HIAA ADDED TO URINE, BY THE COLUMN PROCEDURE

Sample	Total RFI	"Blank" RFI	Corrected RFI	Total μg 5HIAA*	Total μg 5HIAA (average)	Recovery†
5HIAA	3·0	0·066	2·93	1·90		
	3·0	0·039	2·96	1·95	1·93	
Endogenous	1·71	0·34	1·37	0·90		
5HIAA in urine	1·95	0·50	1·45	0·95	0·93	
5HIAA added	4·6	0·48	4·12	2·65		
to urine	4·4	0·35	4·05	2·60	2·63	92%

* This value was obtained after correcting for the dilution factor.
† The recovery of 5HIAA added to urine was calculated as a percentage of the ratio of 2·63:2·86.

TABLE 2. RECOVERIES OF 5HIAA ADDED TO URINE, BY THE BATCH PROCEDURE

Sample	Total RFI	"Blank" RFI	Corrected RFI	Total μg 5HIAA*	Total μg 5HIAA (average)	Recovery†
5HIAA	4·0	0·020	3·98	2·6		
	4·3	0·023	4·28	2·8	2·7	
Endogenous	3·6	0·111	3·49	2·25		
5HIAA in urine	3·3	0·108	3·19	2·10	2·18	
5HIAA added	7·0	0·102	6·90	4·4		
to urine	6·0	0·080	5·92	3·18	3·93	80·5%

* This value was obtained after correcting for the dilution factor.
† The recovery of 5HIAA added to urine was calculated as a percentage of the ratio 3·93:4·88.

DISCUSSION

Methods available for the estimation of 5HIAA in urine are quite plentiful, but the extraction procedures which most of them involve, are tedious at best as volumes of organic solvents have to be handled.[3, 4] These methods also suffer from a lack of specificity as many drugs and their metabolites interfere with the colour reactions of 5HIAA.[6, 8, 10] Preliminary isolation of 5HIAA by paper chromatography[6] before the application of non-specific colour reactions helps to solve this problem, but the extra time involved makes this approach very lengthy, if several routine estimations have to be carried out in a normal working day. The application of fluorimetry to this analysis does not seem to have been investigated. This is surprising, because such a technique would give a very high sensitivity and specificity. Udenfriend *et al.*[11]

have shown the presence of a specific fluorescence peak at 550 mμ for 5-hydroxyindoles in 3N HCl when activated at 295 mμ. The urine, however, contains many highly fluorescing compounds under these conditions, and Fig. 1 shows the fluorescence spectrum of urine in 3N HCl activated at 295 mμ. It can be seen from the same figure that the peak at 535 mμ for pure 5HIAA is masked by urinary contaminants, and these would have to be removed or suppressed to enable use of this peak for 5HIAA estimation. The property whereby 5HIAA is selectively adsorbed on G-10 Sephadex,[7] was made use of for the removal of the interfering fluorophores. Figure 2 shows the 535 mμ peak quite prominently for 5HIAA eluted from a Sephadex column, after urinary contaminants have been washed through. The figure also shows the disappearance of the 535 mμ peak as a result of the oxidation of the 5-hydroxyl (phenolic) group by potassium persulphate. In a previous study,[9] H_2O_2 was used to achieve a similar oxidation of this functional group on the 5HT molecule, but the 5-OH group of 5HIAA was found to be more resistant to H_2O_2 and required the use of the stronger oxidizing agent, potassium persulphate. The normal amount of 5HIAA present in 1 ml of urine is so high, that even as strong an oxidizing agent as potassium persulphate takes some time to effect a complete oxidation. This step is important, as it gives a urine "blank", and the RFI of the solution after the addition of persulphate, is due to non-specific fluorescence unrepresentative of 5-hydroxyindoles.

The recovery of 5HIAA added to urine using the column procedure (Table 1) is higher than that obtained by the batch procedure (Table 2). The latter procedure is, however, reliable for routine estimations and it can be carried out quite comfortably in half an hour. The column procedure does not take much longer and has the advantage of a more reproducible and accurate result. Also, the Sephadex columns can be used repeatedly, the only preparation needed is the washing of the column with 20–25 ml of 0·1N HCl before the transfer of the urine mixture. This procedure has therefore been adopted in our laboratories.

The specificity of this method excludes all compounds other than 5-hydroxyindolic acids, as under the conditions of column chromatography, the basic and neutral aromatic compounds are washed through leaving the aromatic acids adsorbed.[7] Further specificity is afforded by using the 5-hydroxyindole peak at 535 mμ in the fluorescence analysis. 5-hydroxyindolepyruvic and 5-hydroxyindolelactic acids may be included in this assay; however, the former is very unstable and therefore unlikely to be present in urine, and the latter has not as yet been isolated from human urine.

The method can easily be scaled down tenfold or more if required, and indeed we are using it at present for investigations into the 5HIAA content of urine from 12- to 16-week foetuses, obtained after hysterotomies. The volume of such urine varies from 0·2 to 0·8 ml. The method is also being applied to ascertain the presence or absence of foetal 5-hydroxytryptophan decarboxylase; by looking for ^{14}C-5HIAA in foetal urine, after the injection of ^{14}C-5-hydroxy-D,L-tryptophan into the umbilical vein of the foetus during hysterotomy.

Our results (unpublished) have shown maximum recoveries of 5HIAA to be obtained from 24 hr urines if no preservative or acid is added to the urine container. In other words, 5HIAA seems to be most stable at neutral pH.

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