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DETERMINATION OF 5-HYDROXYINDOLEACETIC ACID IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: A FULLY AUTOMATED METHOD

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SUMMARY

High-performance liquid anion-exchange chromatography is combined with continuous flow spectrofluorometry at an optimalized pH for the determination of 5-hydroxyindoleacetic acid (5-HIAA) in urine. Its chief advantages are speed (7 min per determination), enabling measurement of large series of samples, precision (coefficient of variation = 0.72%), sensitivity (detection limit: 1 pmol) and specificity (no interference with 5-HIAA precursors or with the drugs tested). Reference values ($n = 112$) are 25 ± 20 μmol per 24 h. Comparison with a reference spectrofluorometric technique shows a correlation coefficient of 0.96.

INTRODUCTION

5-Hydroxyindoleacetic acid (5-HIAA), a major serotonin catabolite, is eliminated in the urine. In carcinoid tumours, 95% of which occur in the small intestine, very large quantities of serotonin are secreted by the enterochromaffin cells. Early detection of these tumours is essential since unless surgically treated in the initial stages, their prognosis is serious. Such detection is ensured by determination of blood serotonin and urinary 5-HIAA[1].

Systematic measurement of urinary 5-HIAA calls for a quick, reliable and automated technique. For this purpose, the α-nitrosonaphthol colorimetric method lacks specificity[2] and spectrofluorometry after ethyl acetate extraction is time-consuming and tricky[1,3]. Both these methods are manual. Low pressure chromatography techniques are time-consuming (5-HIAA retention time >30 min) and either insensitive or unreliable[4–6]. Reversed-phase separation chromatography also lacks sensitivity and specificity[7], except if preceded by manual extraction which is often time-consuming and reduces the overall precision. Recently, a new electrochemical detection method[8] has been described for tryptophan metabolites at the picomole level. However, 5-HIAA in urine must initially be isolated on extraction columns, eluted with a suitable solvent and then injected into a liquid chromatograph connected to an amperometric detector. This general approach might be of great interest in a research laboratory but not in routine clinical investigation. On the other hand, high-

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performance liquid chromatography (HPLC) allows finer separation but, for the principal methods described[9,10], prior extraction is always required especially when these methods are applied to 5-HIAA determination in urine. One technique[11] also uses UV detection which is not highly specific for 5-HIAA measurement.

We propose a fully automated method in which 5-HIAA is separated from the other metabolites by anion-exchange HPLC at pH 2.30; this permits fast, efficient separation. Continuous flow spectrofluorometric detection is carried out at pH 7.0, at excitation and fluorescence wavelengths of 301 and 338 nm respectively. Chromatography and detection are continuously coupled, in accordance with a process which was developed in our laboratory[12,13] and permits optimization of 5-HIAA pH readings. The use of an automated injector increases precision and allows large numbers of samples to be handled.

MATERIALS AND METHODS

Apparatus

A Chromatem 38 (Touzart Matignon) high-performance liquid chromatograph was connected to an AutoAnalyzer II peristaltic pump, fluorometer and recorder (Technicon) and Micromeritics 725 automated sampler and injector (Coultronics). A Whatman 10-μm Partisil SAX, column (250 × 4.7 mm I.D.) was used with a precolumn (150 × 4.7 mm).

Reagents and standard

The mobile phase, $5 \cdot 10^{-3}$ *M* $NH_4H_2PO_4$ adjusted to pH 2.30 with concentrated H_3PO_4, and the phosphate buffer solution, pH 7.0, 0.1 *M* (8.7 g/l $Na_2HPO_4 \cdot 2\,H_2O$ + 5.6 g/l KH_2PO_4), are stable for 3 months at +4°C. A sulphosalicylic acid solution (500 g/l) was prepared in distilled water. The 5-HIAA standard, 250 μM in 0.01 *M* HCl, is stable in a brown flask for 2 weeks at +4°C. It was prepared from a stock solution containing 2.5 m*M* 5-HIAA in 0.01 *M* HCl which is stable for 3 months at +4°C in a dark bottle. The internal standard, 2 m*M* N-acetyltryptophan (NAT) solution in 0.01 *M* HCl, may be kept in a brown flask for 3 months at +4°C.

Preparation and analysis of urine samples

Urine was collected over a 24 h period in 5 ml HCl (10 *M*). During the 24 h preceding this collection, the subjects were asked to not to eat foodstuffs such as

TABLE I

SAMPLING CONDITIONS FOR AN AUTOMATIC INJECTOR

Vortex stir for 15 sec.

	Volume (µl)			
	External standard	*Urine*	*Urine + 25 µM 5-HIAA*	*Urine + 50 µM 5-HIAA*
NAT (2 m*M*) (internal standard)	100	100	100	100
5-HIAA (250 μM)	50	—	50	100
Mobile phase	550	100	50	—
Acidified urine	—	500	500	500

bananas and grapefruits and to avoid drugs such as reserpine, fluorouracil, heparin, isoniazide, methyldopa, monoamine oxydase inhibitors, corticotrophin and metamphetamine, which affect the *in vivo* urinary concentration of 5-HIAA. A 5-ml volume of urine was acidified by adding 50 μl of sulphosalicylic acid solution. The preparation was then vortexstirred for 15 sec, centrifuged when necessary and filtered.

Determinations were made as follows. For standardization, each subject's urine was submitted to double overloading with known amounts of 5-HIAA in the presence of the internal standard (NAT). An external standard was used for each series of determinations for the purpose of verifying the fluorescence yield of overloading. When an automatic injector is available, the amounts indicated in Table I can be introduced directly into the sampling vials. A 50-μl volume of each sample was automatically injected every 7 min. The apparatus is represented in Fig. 1. The flow-rate was 1.50 ml/min, and an example of a urinary chromatogram is given in Fig. 2.

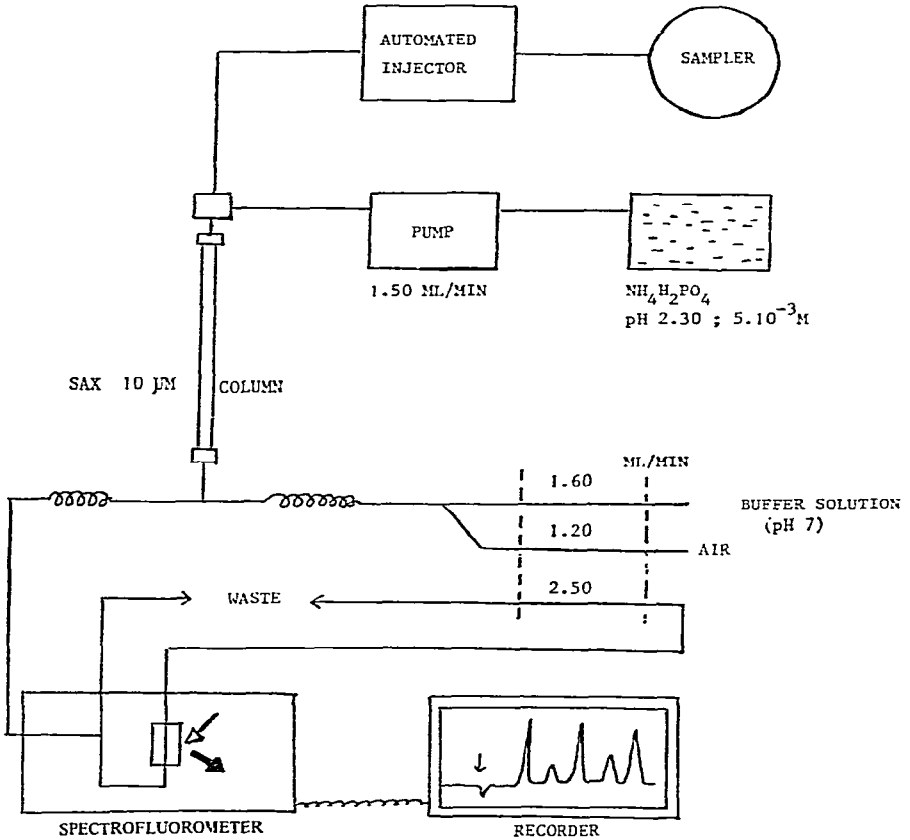

Fig. 1. Chromatographic and analytical system for fully automated determination of 5-HIAA, $\lambda_{ex.} = 301$ nm; $\lambda_{em.} = 338$ nm.

RESULTS AND DISCUSSION

Choice of experimental conditions

The pH and the concentration of the mobile phase were varied within limits

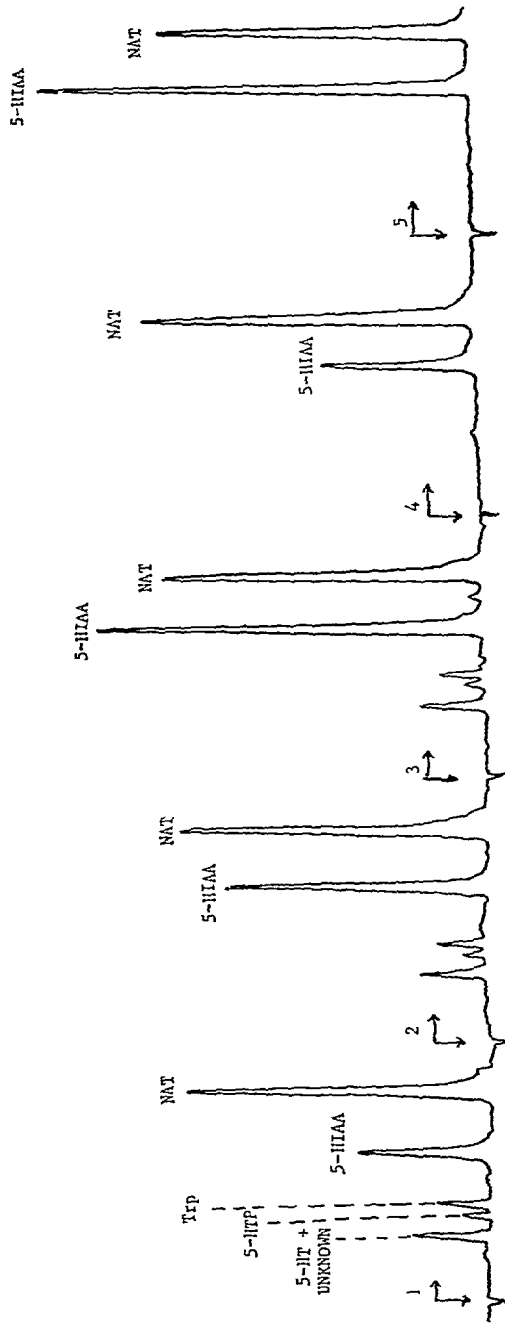

Fig. 2. Chromatograms of urine (1), urine + 25 μM 5-HIAA (2), urine + 25 μM 5-HIAA (3), 25 μM 5-HIAA standard (4) and 1 μl of urine from subject with carcinoid tumour (5).

compatible with the stationary phase, *i.e.*, from pH 1.50 to 7.00 and from 10^{-4} *M* to 1 *M*. Under the optimal conditions of pH 2.30 for a concentration of $5 \cdot 10^{-3}$ *M* 5-HIAA was well separated from its metabolites (tryptophan Trp; hydroxy-5-tryptamine 5-HT; hydroxy-5-tryptophan, 5-HTP) (Fig. 2).

The fluoroscence spectrum of 5-HIAA was verified with our apparatus and the excitation and emission maxima were found to be 301 and 338 nm respectively. The effect of pH was studied between 2 and 10, and the optimal pH was found to be 7.0, at which the fluorescence intensity of 5-HIAA was four times that at pH 2.30.

Chromatography requires the use of an internal standard to eliminate uncertainty regarding the volumes injected. The spectrofluorometric characteristics of NAT enable satisfactory detection under the conditions described above. Its retention time is 1 min 30 sec longer than that of 5-HIAA.

Fluorometric detection requires standardization by overloading. The overloading yield (R) was established for each determination. It enabled us to determine the presence of either a fluorescence activator ($R > 100\%$) or inhibitor ($R < 100\%$). In 99% of cases, the overload yield was between 90 and 105%. Minor interferences were then automatically corrected by calculating the overload.

Analytical results

The detection limit was 1 pmol, in accord with published results[6,7,9]. Linearity was satisfactory up to 250 μM, *i.e.*, about ten times the usual mean value. As regards within-run precision, 36 successive measurements of the same sample (28.5 μM) yielded the following coefficients of variation (C.V.): 2.90% for manual injection; 1.30% for automatic injection with no internal standard and 0.72% for automatic injection with an internal standard.

Sample preparation is simple and quick (a few minutes) and one sample can be injected every 7 min. Since injection is automatic, the apparatus requires no supervision.

The absence of contamination was verified by measuring low standards (5 μM) and high standards (100 μM). There was no significant difference ($p < 0.05$) between the low standard values, whether they preceded or followed the high concentrations.

The specificity of the 5-HIAA determinations was ensured by combining chromatographic separation with spectrofluorometric detection: chromatography permitted separation of 5-HIAA from its metabolic precursors present in urine, *i.e.*, Trp, 5-HTP and 5-HT (Fig. 2); spectrofluorometry was carried out at the wavelengths of the maxima and at the optimum pH for 5-HIAA.

The extent of interference was tested on certain drugs, particularly those interfering in the α-nitrosonaphthol technique. In each test the drug was added to urine at a concentration corresponding to the maximum therapeutic dose; 6 g/l acetylsalicylic acid; 150 mg/l promethazine; 200 mg/l chlortetracycline; 2.5 g/l sulfamethoxazole; 150 mg/l chlorpromazine and 100 mg/l prochlorperazine. At these concentrations, no significant interference was shown, unlike that observed with the colorimetric method.

In order to compare our direct method with a spectrofluorometric reference method, 68 determinations of urinary 5-HIAA were made by a manual spectrofluorometric technique used in our laboratory[3], preceded by extraction. A statistical

study showed a good correlation: correlation line, $y = 0.89\,x + 1.88$; correlation coefficient, $r = 0.96$; t (Student) $= 38.9$; theoretical $t = 3.44$ ($p < 0.001$). Reference values, determined in 112 healthy subjects, were 25 ± 20 μmol/24 h (mean $\pm$ 2 σ, $p < 0.005$), in accord with published values[1,2,4,5,14–17].

Urinary 5-HIAA was also determined in nineteen cases of carcinoid tumours. Values ranged from 94 to 4350 μmol per 24 h. A typical chromatogram is shown in Fig. 2. Results were excellently correlated with the reference technique[3] and agreed with clinical and anatomopathological observations.

CONCLUSION

High-performance liquid chromatography combined with continuous flow fluorometric detection at an optimized pH permits quick and specific measurement of urinary 5-HIAA. Since the method is both completely automated and simple, it can be used for systematic determinations in carcinoid tumour detection. Its specificity enables ethyl acetate extraction to be avoided and its precision and sensitivity make it a particularly reliable method for use in clinical biochemical laboratories.

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