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Note

Simultaneous automated high-performance liquid chromatographic determination of 5-hydroxy-3-indoleacetic acid and homovanillic acid in urine with fluorescence detection

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Intestinal carcinoid tumors, which secrete large amounts of serotonin, are often discovered by the enhanced excretion of 5-hydroxy-3-indoleacetic acid (5-HIAA) 5-HIAA determination has also been suggested as an aid in the diagnosis of certain neurological [1,2] and psychiatric disorders [3,4] Homovanillic acid (HVA) is the main metabolite of dopamine in the brain Neuroblastomas and ganglioneuromonas are generally accompanied by increased levels of HVA [5]. Interactions between the dopaminergic and serotonergic systems have been assumed [6]. However, to our knowledge, no simple method for the simultaneous determination of 5-HIAA and HVA in urine has been reported

Most of the published methods for monoamine determinations concentrated on high-performance liquid chromatography (HPLC) with electrochemical detection and/or required sample purification (e g , ion-exchange chromatography, solvent extraction), easy matrices such as cerebrospinal fluid were used or complex methodology, including gradient systems, were described All publications in the field of monoamine determinations agree on the difficult aspect of the complexity of the urine sample In this paper, we present a simple automated HPLC method for the simultaneous measurement of HVA and 5-HIAA in urine without sample pretreatment

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EXPERIMENTAL

Reagents and materials

Ethylenediaminetetraacetic acid sodium salt (EDTA) and sodium dihydrogenphosphate were obtained from Merck (Darmstadt, F R G) and tetramethylammonium hydrogensulphate from Fluka (Buchs, Switzerland) 3,4-Dihydroxymandelic acid, 3,4-dihydroxyphenylacetic acid, L-3,4-dihydroxyphenylalanine (L-DOPA), 3,4-dihydroxyphenyl glycol, dopamine, homogentisic acid, HVA, 5-HIAA, 5-hydroxy-2-indolecarbonic acid, hydroxy-3-methoxyphenylethylene glycol (MOPEG), 5-hydroxytryptophan, 3-indoleacetic acid, kynurenine, norepinephrine, normetanephrine, serotonin, tyramine, tyrosine, tryptamine, tryptophan, vanillic acid and vanillylmandelic acid (VMA) were purchased from Sigma (Deisenhofen, F R G) Lyphochek control urines were obtained from Bio-Rad Labs (Munich, F R G)

Stock solutions of reference compounds were prepared in methanol and stored at 4°C Working solutions were freshly prepared every day

Apparatus

The chromatographic analysis was performed with two 655A-12 liquid chromatography pumps, an L5000 LC controller, a D2000 integrator (Merck) and an EL7000 electromagnetic valve (Krannich, Gottingen, F R G) Analyses were carried out on a 25 mm×4 mm I D LiChrospher RP-18e pre-column (end-capped, 5 μm) and a 125 mm×4 mm I D LiChrospher RP-18e analytical

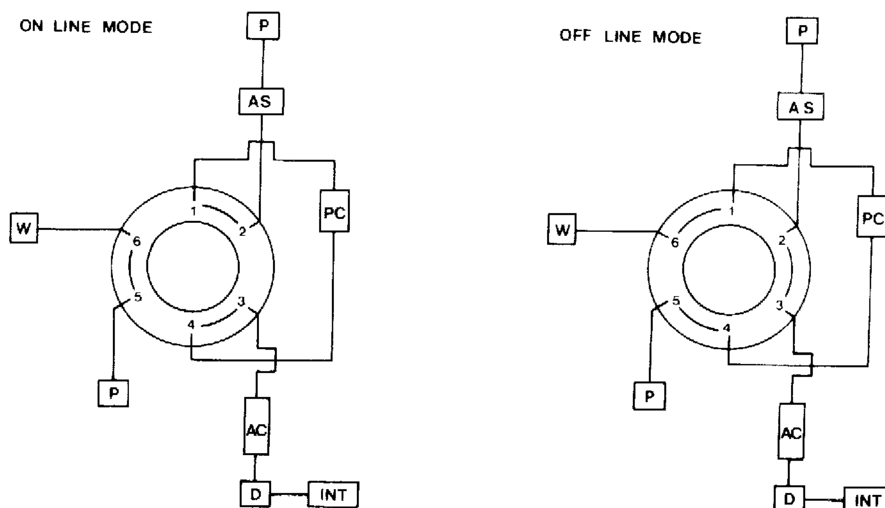

Fig 1 Diagram of the HPLC column-switching system in on-line and off-line modes AC = analytical column, AS = autosampler, D = fluorescence detector, INT = integrator, P = pump, PC = pre-column, W = waste

column (end-capped, 5 μm) (Merck) An F1000 fluorimetric detector (Merck) with a 40-μl flow cell set at an excitation wavelength of 275 nm and an emission wavelength of 330 nm was employed The system was automated by a 655A-10 autosampler (Merck) The flow diagram of the column-switching system is given in Fig 1

Chromatographic conditions

The aqueous part of the mobile phase was prepared by adding 0 2 g of EDTA, 13 8 g of sodium dihydrogenphosphate and 0 7 g of tetramethylammonium hydrogensulphate to deionized water to a final volume of 1 l and adjusted to pH 5 4 with 13 *M* ammonia The eluent was buffer–methanol (95 5, v/v) The flow-rate was 1 2 ml/min

Column-switching system

The configuration of the column-switching apparatus in on-line and off-line modes is given in Fig 1 In the on-line mode the urine samples were injected via an autosampler and were preseparated from lipophilic impurities on a pre-column For 2 min all hydrophilic compounds, including 5-HIAA and HVA, eluted from the precolumn and passed for subsequent separation to an analytical column After valve-switching the system went into the off-line mode and all lipophilic urine constituents retarded on the pre-column were eluted in the back-flushing mode for 8 min with the same eluent The cycle time for one analysis was 12 min The integrator, electromagnetic valve and autosampler were controlled by the L5000 LC controller

Sample preparation

Urine samples for analysis were collected over a 24-h period in polyethylene containers and 10 ml of 8 *M* hydrochloric acid were added as a preservative Samples were stored at −20°C until analysis Before analysis the thawed samples were vortex-mixed and centrifuged at 10 000 *g* for 10 min at 8°C A 50-μl sample of the urine supernatant was diluted with 1 5 ml of HPLC buffer and 20-μl aliquots were injected at 4°C via an autosampler Calibration was performed with stock solutions, freshly diluted with the HPLC buffer and checked periodically with Lyphochek control urines

RESULTS

To achieve the separation of 5-HIAA and HVA from other co-eluting substances without extraction procedures and without increasing the total analysis time, the column-switching technique was useful Under the described optimum assay conditions, retention times and corresponding relative retention times calculated on 5-HIAA were measured (Table I) HVA and 5-HIAA are well separated, and also serotonin and homogentisic acid (see Fig 2A)

TABLE I

RETENTION TIMES (RT) OF BIOGENIC AMINE METABOLITES AND INTERFERING COMPOUNDS

All relative retention times (RRT) are quoted relative to 5-HIAA

Substance	RT (min)	RRT	Substance	RT (min)	RRT
3,4-Dihydroxymandelic acid	0 99	0 14	5-Hydroxytryptophan	4 50	0 64
Norepinephrine	1 02	0 15	Vanillic acid	4 70	0 67
VMA	1 30	0 19	MOPEG	5 14	0 74
L-DOPA	1 44	0 21	Serotonin	6 00	0 86
Normetanephrine	1 58	0 23	5-HIAA	6 98	1 00
Tyrosine	1 90	0 27	HVA	8 13	1 16
3,4-Dihydroxyphenyl glycol	2 00	0 29	Tryptamine	9 40	1 35
Dopamine	2 13	0 31	Kynurenine	9 42	1 35
3,4-Dihydroxyphenylacetic acid	2 65	0 38	Tryptophan	9 70	1 39
Homogentisic acid	2 90	0 42	5-Hydroxy-2-indolecarbonic acid	11 52	1 65
Tyramine	3 40	0 49	3-Indoleacetic acid	14 59	2 09

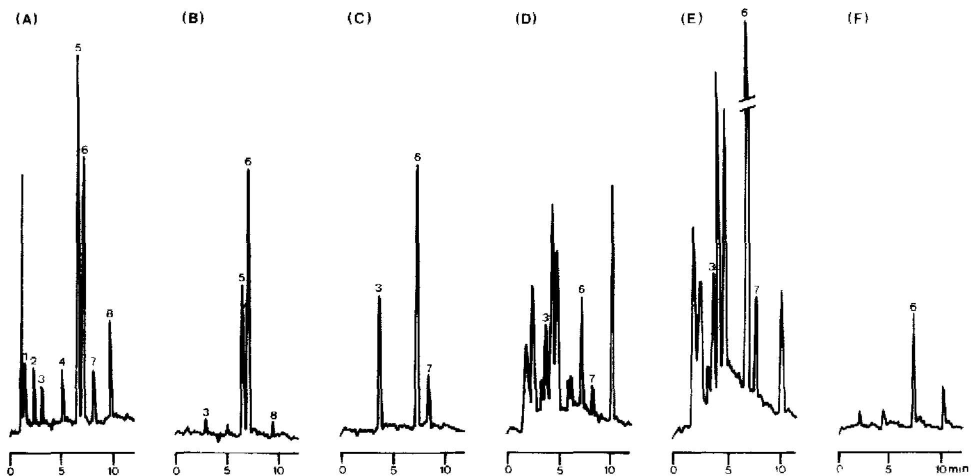

Fig 2 HPLC traces for different samples (A) Aqueous mixture (5 mg/ml of each compound) measured at 275/330 nm, (B) aqueous mixture (5 mg/ml of each compound) measured at 300/355 nm, (C) external calibration sample of homogentisic acid (20 mg/ml), 5-HIAA (5 mg/ml) and HVA (5 mg/ml) measured at 275/330 nm, (D) normal urine sample measured at 275/330 nm (5-HIAA 2 23 mg/l and HVA 3 07 mg/l), (E) abnormal elevated urine sample measured at 275/330 nm (5-HIAA 15 28 mg/l and HVA 8 85 mg/l), (F) normal urine sample (D) measured at 300/355 nm Peaks 1 = VMA, 2 = dopamine, 3 = homogentisic acid, 4 = vanillic acid, 5 = serotonin, 6 = 5-HIAA, 7 = HVA, 8 = tryptophan

Serotonin, with a much lower physiological urine level than the compounds of interest, cannot be determined with this HPLC assay system at normal physiological urine concentrations, and is measurable only with abnormally high urine levels

This automated HPLC system for simultaneous 5-HIAA and HVA determinations is advantageous because other urinary physiological metabolic compounds are detectable in a single run and may give indications of other metabolic disorders or diseases Homogentisic acid is well separated from other urinary constituents (see Fig 2) and can easily be detected by this method In principle, this compound is measurable by the described method but its determination is hardly demanded in clinical laboratories Abnormally high levels of homogentisic acid were found in alkaptonuria, a rare metabolic disorder

Tryptophan, with a retention time of 10 41 min, is well separated from HVA A problem in its determination in urine is the presence of other possible endogenous interferents such as tryptamine and kynurenine Therefore, no exact tryptophan determination is possible Despite this fact, a metabolic disorder in this pathway would be indicated by very high peak signals

Another interesting compound is VMA, with a retention time of 1 3 min Only high levels of VMA would be recorded because other unknown urine constituents interfere with this peak However, for these two compounds, tryptophan and VMA, this automated HPLC method may serve as an indicator for further investigations when high signals are observed in routine 5-HIAA and HVA analysis

5-HIAA and HVA were detected selectively by fluorescence detection, which was demonstrated with chosen excitation and emission wavelengths (Fig 2A and B) 5-HIAA showed nearly the same signal intensity at both settings whereas HVA, tryptophan and homogentisic acid revealed 50-, 10 6- and 3 7-fold higher intensity, respectively, at 275-nm excitation and 330-nm emission wavelengths in comparison with 300/355 nm Norepinephrine, VMA and dopamine were not detectable at 300/355 nm

Fig 2D and E show typical chromatograms with normal and elevated 5-HIAA concentrations, the latter having been caused by a carcinoid tumor In Fig 2F the same analysis as in Fig 2D was carried out at 300-nm excitation and 355-nm emission wavelengths This demonstrated that selectivity may be favourable for abnormally high sample compound levels for control purposes

Calibration of the HPLC system was performed with aqueous dilute standards and controlled with Lypochek urines For linearity, recovery and precision studies on 5-HIAA and HVA, the standard addition method was applied to a pooled urine sample Good linearity was observed in the range 0 5–20 mg/l Assuming a signal-to-noise ratio of at least 3, the detection limits of the assay were 0 2 and 0 5 mg/l for 5-HIAA and HVA, respectively Recoveries of 5-HIAA and HVA in urine at concentrations of 2, 4 and 6 mg/l for each analyte were 98 3 ± 2 3 and 97 1 ± 3 9%, respectively The intra-assay ($n = 10$) and in-

ter-assay ($n=9$) relative standard deviations for spiked urine samples at a level of 4 mg/l for each analyte were 2 9 and 4 2% (5-HIAA) and 2 5 and 3 9% (HVA)

DISCUSSION

The described HPLC method provides a simultaneous, automated, rapid and sensitive determination of 5-HIAA and HVA by a column-switching technique No sample pretreatment was required and the use of fluorescence detection simplified its use in comparison with the more troublesome electrochemical detection Recent publications on HVA and 5-HIAA determinations described electrochemical detection in combination with urine extraction procedures such as ion-exchange [7], gel permeation [8] or solvent–solvent extraction [9,10] Further, some of these HPLC systems required long analysis times of 30–60 min [8,10] Only one publication reported direct urine injection [11], but one analysis required 30 min and the complexity of the urine sample was emphasized One advantage of the column-switching technique is the use of the black-flushing mode, which elutes strongly retarded urine constituents during the analysis and as a consequence no interference problems arose and the analysis time was shortened

In conclusion, the proposed method improved the conditions for the routine clinical determination of 5-HIAA and HVA This system has been in operation for over two years without disturbances and with a minimum of technical attendance

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