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SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE CONCURRENT DETERMINATION OF THE AMINE METABOLITES VANILLYLMANDELIC ACID, 3-METHOXY-4-HYDROXYPHENYLGLYCOL, 5-HYDROXYINDOLEACETIC ACID, DIHYDROXYPHENYLACETIC ACID AND HOMOVANILLIC ACID IN URINE USING ELECTROCHEMICAL DETECTION

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SUMMARY

A simple method for the concurrent analysis of the noradrenaline metabolites vanillylmandelic acid and 3-methoxy-4-hydroxyphenylglycol, the dopamine metabolites dihydroxyphenylacetic acid and homovanillic acid, and the serotonin metabolite 5-hydroxyindoleacetic acid in human urine is described. Following organic extraction of the metabolites from acidified urine, they are separated by single-step gradient elution high-performance liquid chromatography on a reversed-phase column. Detection and quantification are achieved with an electrochemical detector using a carbon-paste electrode; samples can be injected at 40-min intervals. Optimisation of analytical parameters is described, and examples of the application of the method in the fields of clinical chemistry and clinical neuroscience are given. This provides a convenient method for the concurrent study of the metabolism of three major biogenic amines, and is readily adaptable for studies on cerebrospinal fluid and brain tissue.

INTRODUCTION

The urinary excretion of the metabolites of catecholamines and 5-hydroxytryptamine is used in screening for a variety of disease states including phaeochromocytoma, neuroblastoma and carcinoid syndrome [1, 2]. In particular, the ability to detect elevated dopamine as well as noradrenaline metabolites is of considerable clinical significance in assessing the malignancy of phaeochromocytoma [3].

Traditional methods for these determinations, usually spectrophotometric, have lacked sensitivity and specificity. Normal variations in the excretion of

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these compounds, or smaller deviations therefrom, are of interest in the context of research in neurology and psychiatry (see ref. 4, for example). This has led to the development of much more specific and reliable methods which, however, require expensive equipment (for example, gas chromatograph—mass spectrometer) and measure one or at most two metabolites of interest.

High-performance liquid chromatography (HPLC) on microparticulate reversed-phase columns offers a powerful technique for separating related compounds which are water-soluble but with some hydrophobic character. UV-absorption detection lacks the requisite sensitivity for the measurement of endogenous amine metabolite levels. Electrochemical (EC) detection offers a major increase in sensitivity, and is also somewhat selective for these metabolites.

Recently HPLC—EC methods for vanillylmandelic acid (VMA) [1], 3-methoxy-4-hydroxyphenylglycol (MHPG) [5], dihydroxyphenylacetic acid (DOPAC) [6], homovanillic acid (HVA) [7] and 5-hydroxyindoleacetic acid (5HIAA) [8] have all been described. This paper presents a simple method for the determination of all five metabolites following organic extraction from acidified urine, using single-step gradient chromatography. Samples can be injected at 40-min intervals; the simplicity of the apparatus and the procedure makes the method suitable for clinical laboratory screening as well as for clinical neuroscience research purposes.

EXPERIMENTAL

HPLC apparatus

This was made up from modular components supplied by Anachem (Luton, Great Britain) as follows: Altex 110A single-piston pump, external flow through pulse dampener; Rheodyne 7120 injection valve equipped with 20-μl sample loop; 15 cm × 4.6 mm column packed with Hypersil ODS 5 μm, at ambient temperature; Bioanalytical Systems (BAS) electrochemical detector LC-12 packed with CP-O carbon paste with LC-2A or LC-4 control box, reference electrode Ag/AgCl (RE1); Bryans 28000 series, single-pen recorder; for solvent switching an Altex slider valve adapted for automatic operation using two pneumatic activators and solenoid air valves, with timing device based on motor-driven cams operating microswitches (Radiospares).

Materials

The HPLC solvent was 0.1 *M* (final) sodium phosphate buffer (pH 3.0) containing 5 or 15% AnalaR methanol (BDH, Poole, Great Britain). Ethyl acetate (special for chromatography) and other reagents (AR where available) were obtained from BDH. Standards of the amine metabolites were obtained from Sigma (Poole, Great Britain). Working standard solutions contained VMA, 5HIAA, DOPAC and HVA, all at 25 μg/ml and MHPG at 50 μg/ml (as free glycol). β-Glucuronidase (Bacterial type II 48,000 units/g) was obtained from Sigma and arylsulphatase from Boehringer (London, Great Britain).

Sample preparation

Enzymatic hydrolysis of conjugated metabolites (for total metabolite determination) was achieved as previously described [4] by incubation of 0.45 ml of urine with 50 µl of water or mixed standard, 80 µl of 1 mol/l Tris—acetate buffer (pH 6), 25 µl of β-glucuronidase (100 mg/ml, freshly prepared) and 15 µl of arylsulphatase at 37°C for 16 h.

The incubate (or, for free metabolites, urine with added buffer, and water in place of the enzymes) was adjusted to pH 1 by the addition of 30 µl of concentrated HCl. The metabolites were then extracted successively with 1.5- and 1.0-ml portions of ethyl acetate, 1.2 and 1.0 ml, respectively, of the organic phase being recovered.

Solvent was removed from the pooled extracts at room temperature under a stream of oxygen-free nitrogen. Residues were taken up in 0.5 ml of distilled water for chromatography, and stored at —40°C where necessary.

HPLC conditions

The buffer flow-rate was 1 ml/min, the EC detector voltage 0.72 V (vs. Ag/AgCl reference), sensitivity was 100 nA f.s.d., and injection volume 20 µl. The 5% methanol buffer was automatically switched to 15% methanol buffer 5.5 min after injection (this solvent front reached the detector 8 min later); 24 min after injection the solvent was switched back to 5% methanol and the next sample could be injected 15 min later. Traces were recorded at 1 V f.s.d. and quantification was by peak height measurement, using the method of standard additions.

Analysis of an enzyme blank confirmed that none of the peaks on the chromatograms arose from the added enzymes.

RESULTS AND DISCUSSION

Method development

In preliminary experiments using relatively large amounts (20 µg) of catecholamine metabolite standards and a Cecil UV detector at 280 nm it was established that VMA, MHPG, DOPAC and HVA could be well resolved on a 15-cm column of Hypersil ODS 5 using McIlvaine buffers (0.2 *M* Na_2HPO_4 + 0.1 *M* citric acid, pH 2.7). The retention times could be controlled by varying the pH or concentration of added methanol. Using the EC detector at 0.72 V, 20-ng samples were readily quantified, an increase of 1000-fold in sensitivity over the UV detector. (Catechol and hydroxyindole compounds can be detected at ca. 0.5 V; the higher voltage is necessary to detect the methoxyhydroxy derivatives.)

Increasing the methanol concentration (Fig. 1), or increasing the flow-rate, reduced the retention times for all five metabolites. Increasing the pH of the eluting buffer at 10% methanol reduced the retention of the acidic metabolites (by shifting the equilibria towards the ionised species), but not that of the neutral metabolite MHPG (Fig. 1). The anomalous effects of pH on MHPG and DOPAC at low methanol concentrations may well be due to interactions with the ionic species present in the buffer. This was also sug-

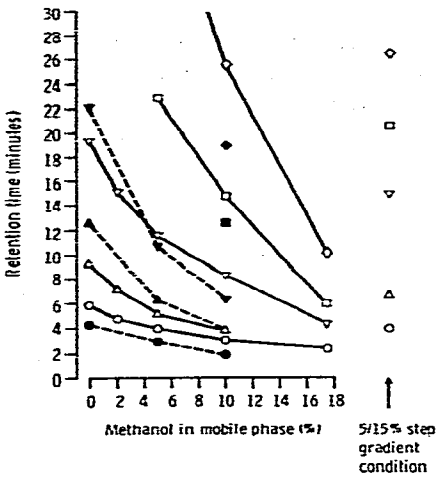

Fig. 1. Effect of added methanol on retention times. Standards of VMA (○ ●), MHPG (△ ▲), DOPAC (▽ ▼), 5HIAA (□ ■) and HVA (◇ ◆) were chromatographed using citrate—phosphate buffers (see text) at pH 2.7 (open symbols) and pH 4.0 (closed symbols) with increasing concentrations of methanol as indicated. Retention times for the final conditions with phosphate buffer (pH 3) and a step gradient of methanol are indicated at the right.

gested by the increased retention times found when 0.1 *M* phosphate buffer was substituted for citrate—phosphate (because of a report [9] that citrate ions had a detrimental effect on the life of reversed-phase columns).

Chromatography of urine extracts in 0.1 *M* phosphate buffer (pH 3) yielded peaks corresponding to the five metabolites. Addition of 5% methanol to this buffer reduced the retention time of HVA, the last peak to elute, from 96 to 60 min; further increases of methanol resulted in a loss of resolution in the area of VMA and MHPG. Variation of pH and methanol in a manner analogous to that in Fig. 1 did not yield suitable isocratic conditions, and thus a single-step gradient elution using 5 and 15% methanol was adopted; the resultant retention times are also shown in Fig. 1. The conditions described under Methods enabled a complete analysis to be made within 40 min with good resolution of the peaks of interest.

The change of solvent during the analysis resulted in a hump on the baseline; this was due to the 2-μm stainless-steel inlet filter on the line in which the second solvent stood before the switch. Changing the filters to sintered glass resulted in the abolition of the baseline disturbance (see Figs. 3 and 4). Recovery of standards added to urine was of the order of 120%, i.e. more was recovered when taken through the method in urine than in an aqueous solution. Salt saturation of aqueous standards did not improve recovery, and thus the method of standard addition (spiking) was used for each urine. The amount added was about one-third of the normal urinary concentration (see Fig. 3). Recovery between urine samples was consistent (Table I), so that a few spiked urines could be used to quantitate all those analysed in one series.

Application of the method

Using the method as described, VMA, MHPG, DOPAC, 5HIAA and HVA can be determined following a single HPLC injection using the EC detector at the least sensitive setting of 100 nA f.s.d. A linear relationship was obtained (Fig. 2) between detector response and increasing amounts of standards added to urine samples before extraction. Table I shows the recoveries through the extraction of standards added to urine (i.e. as a percentage of aqueous standards directly chromatographed). A typical trace from a urine specimen from a normal control subject is shown in Fig. 3. The average deviation from the mean of samples analysed in duplicate ranged from 6.3 to 8.8% for the various metabolites (mean of 15 pairs of determinations).

Fig. 4 shows a trace from a urine specimen from a patient with phaeochromocytoma (subsequently histologically confirmed). VMA excretion was 20.6 mg per 24 h in agreement with the clinical chemistry laboratory

TABLE I

APPARENT RECOVERIES FROM URINE

The data represent recoveries of standards added to urine relative to unextracted standards. Results are expressed as mean ± S.D. of four observations for each metabolite. The results are not corrected for incomplete recovery of organic phases; true recoveries can be obtained by dividing means by 0.9.

Metabolite	Recovery
VMA	72.5 ± 7.3%
MHPG	56.8 ± 6.6%
DOPAC	80.1 ± 8.1%
5HIAA	61.2 ± 7.0%
HVA	74.6 ± 11.5%

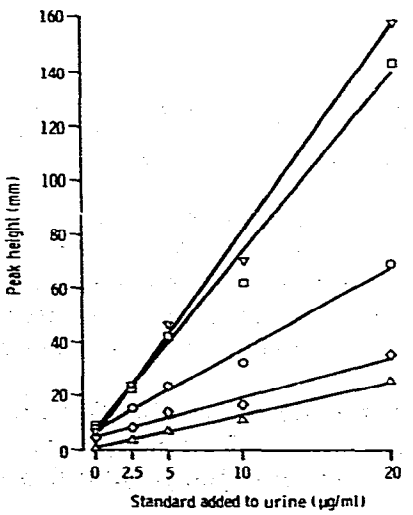

Fig. 2. Effect of adding varying concentrations of standard to a urine sample before extraction and chromatography. Symbols as in Fig. 1.

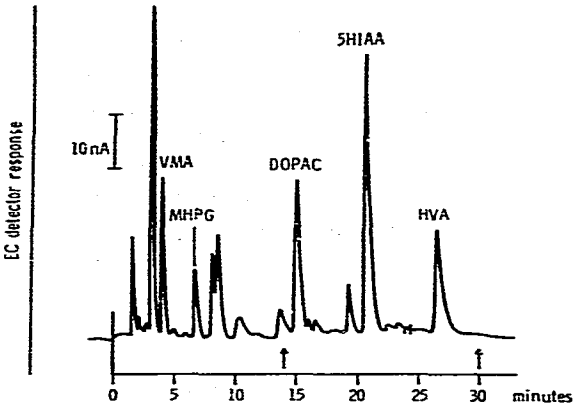

Fig. 3. Chromatogram of a urine extract from a normal subject. Unhydrolysed urine was spiked with standards of approximately one-third of the concentration of each metabolite (except MHPG, which is largely conjugated). Chromatographic conditions: 150 × 4.6 mm column of Hypersil ODS 5; solvent 1 ml/min of 0.1 *M* sodium phosphate (pH 3.0), initially 5% methanol. First arrow: same buffer but with 15% methanol arriving at detector following switch. Second arrow: return to initial conditions.

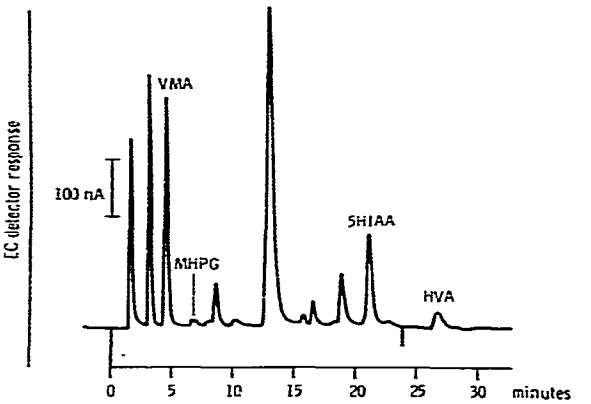

Fig. 4. Chromatogram of urine extract from a patient with phaeochromocytoma. Unhydrolysed and unspiked sample. Note change of ordinate scale. Chromatographic conditions as in legend to Fig. 3. Metabolite levels from this patient are given in the text.

result of 24.4 mg per 24 h, and conjugated VMA was present (total VMA 28.7 mg per 24 h). Total MHPG excretion was 15.1 mg per 24 h (free: 2.8 mg per 24 h, a normal proportion). This represents about a six-fold elevation of VMA and MHPG; total metanephrines were 36 mg per 24 h, again about a six-fold elevation. 5HIAA and HVA excretion were also increased to a lesser extent; conjugated HVA was also present.

Fig. 5 shows the urinary excretion (expressed per 48 h) of the five metabolites, free and total, in a subject who received 100 mg of carbidopa three times daily for one week (shown shaded), and the excretion in the week following.

During the carbidopa administration there was an increase in the output of conjugated metabolites; in particular, conjugated VMA, HVA and 5HIAA were detected in substantial amounts. These are normally a very small propor-

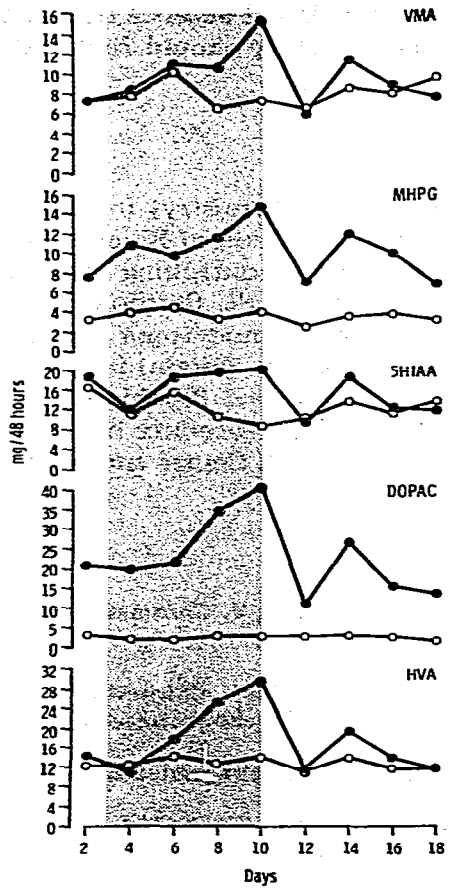

Fig. 5. Effect of carbidopa on excretion of amine metabolites in a normal subject. Carbidopa 100 mg three times daily was administered at 08.00, 14.00, 22.00 h for 7 days (shaded area). Excretion of free (○) and total (= free + conjugated, ●) amine metabolites was determined. Consecutive 24-h urine samples were analysed; results are presented for 48-h periods for clarity.

tion of total output of these compounds. This argues against the conclusion of a previous study [10] which interpreted a reduction in free 5HIAA and in total MHPG excretion in ten subjects on this regimen as indicating effective blockade of extracerebral synthesis of serotonin and noradrenaline.

Simple and cheap modular apparatus can thus be used to give a rapid, accurate and specific determination of the biogenic amine metabolites VMA, MHPG, DOPAC, HVA and 5HIAA in urine. Our columns lasted for more than a year with occasional repacking of the inlet end. The carbon-paste electrodes last from 1—2 weeks to 1—2 months before requiring repacking, probably due to the relatively high methanol concentrations used in part of the running cycle. Glassy carbon electrodes are more stable, and would be preferred in this application where the ultimate in sensitivity is not required.

Similar principles are applicable to the determination of amine metabolites in cerebrospinal fluid [11] and post-mortem brain [12]. Cerebrospinal fluid can be injected directly after a filtration step [13] and for both brain and

cerebrospinal fluid the reduced number of peaks and virtual absence of VMA means that isocratic elution can be used. This methodology should find wide application in both the clinical and the research laboratory.

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