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Note

Isocratic analysis of 3-methoxy-4-hydroxyphenyl glycol, 5-hydroxyindole-3-acetic acid and 4-hydroxy-3-methoxyphenylacetic acid in cerebrospinal fluid by high-performance liquid chromatography with amperometric detection

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The study of cerebrospinal fluid (CSF) amine metabolites is important for the clinical investigation of diseases of the central nervous system (CNS). A direct measure of CNS metabolism is essential to test the hypothesis concerning putative neurotransmitter abnormalities in various neurological and psychiatric diseases. The main metabolites found in detectable amounts in the CSF are 3-methoxy-4-hydroxyphenyl glycol (MHPG), 5-hydroxyindole-3-acetic acid (5-HIAA) and 4-hydroxy-3-methoxyphenylacetic acid or homovanillic acid (HVA). These metabolites are derived from noradrenaline (NA), 5-hydroxytryptamine (5-HT), and dopamine (DA), respectively.

Abnormalities in the amounts of metabolites found in CSF have been observed in various psychiatric and neurological conditions. For instance, decreased 5-HIAA accumulation has been observed mainly within a subgroup of depressions [1]. Low CSF 5-HIAA and HVA concentrations are also found in Parkinson's disease [2] and are consistent with the low DA and 5-HT concentration in the basal ganglia from such patients. Altered 5-HIAA and HVA accumulation has also been reported in presenile dementia of the Alzheimer type [3] and epileptic children [4] although the literature is conflicting.

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Clearly there is a need for a sensitive and accurate method of analysis for catecholamine metabolites in order to obtain useful data in such studies.

High-performance liquid chromatography (HPLC) has been widely used to separate catechol- and indoleamines using reversed-phase ion-pair systems. These are described in recent reviews [5, 6], which also deal with the methods of detection. Electrochemical detection provides a high sensitivity which is essential for the detection of low-level endogenous amine metabolites in CSF. There have been numerous reports on the separation of monoamine metabolites in urine [7], rat brain [8], and CSF [9], but many of these required the use of gradient elution with complex buffers. In these studies extraction of the metabolites was also usually necessary before chromatographic analysis.

This paper describes a simple isocratic method for the separation of the main metabolites using a simple eluent and with minimal sample pre-treatment.

EXPERIMENTAL

Materials and reagents

Reference catecholamine metabolites were obtained from Sigma (Poole, U.K.) and were of the highest purity available. Ammonium acetate and glacial acetic acid were AnalaR grade from BDH (Poole, U.K.). Solutions of reference compounds and the mobile phase were prepared in distilled deionized water. Reference compounds were kept on ice during use and were freshly prepared each day.

Apparatus

A Pye Unicam (Cambridge, U.K.) LC3-XP pump and an LCA15 electrochemical detector (EDT Research, London, U.K.) were used. The detector, which is of the wall jet type, employed a glassy carbon working electrode and an Ag/AgCl reference electrode. Samples were injected via a Rheodyne 7125 injector fitted with a 50-μl loop. The chromatographic column (250 mm × 5 mm) was slurry-packed with ODS Hypersil (5-μm Spherical silica, chemically bonded with a monolayer of octadecylsilyl groups) from Shandon Southern Products (Runcorn, U.K.).

Chromatographic conditions

The column was washed with 40% methanol for 30 min and then equilibrated with the mobile phase consisting of 0.1 *M* ammonium acetate, adjusted to pH 5.15 with 3 *M* acetic acid, and 0.27 m*M* EDTA. Mobile phases of pH values ranging from 4.80 to 5.70 have been investigated for the separation. The flow-rate was 1.5 ml/min and the temperature ambient. The mobile phase was continuously degassed with a stream of helium. The peaks were detected amperometrically at a detector sensitivity of 3 nA. The effect of varying the oxidation potential on sensitivity was investigated at pH 5.15.

Samples

Human lumbar CSF were obtained from patients with primary depression diagnosed by the criteria used in the Psychiatry Department. Lumbar punctures were performed between 8.00 and 10.00 a.m. after at least 10 h of fasting and

bed rest in a standardized manner. Spinal fluid (13 ml) was drawn and immediately placed on ice. Spinal fluid was also obtained from patients receiving routine lumbar punctures having various neurological disorders. This study was approved by the Hospital Ethical Committee.

All samples were divided into aliquots for storage at −70°C. Prior to the analysis an aliquot was ultrafiltered [10] through an Amicon PM 10 membrane (NMW 10,000), and then injected directly into the HPLC system.

RESULTS AND DISCUSSION

High-performance liquid chromatography

There are few HPLC techniques capable of simultaneously separating MHPG, 5-HIAA, and HVA from other endogenous compounds in human CSF [11, 12]. One of the major problems is that column efficiency is often poor when traditional buffers, such as phosphate buffers, are used. To overcome this problem acidic amine buffers have been suggested [13] as alternatives for the separation of catecholamine metabolites.

It has been shown [14] that ammonium acetate is a superior masking agent for the residual silanol groups of reversed-phase packings leading to greatly improved column efficiency. It is also compatible with electrochemical detection, being an electrolyte with relatively high electrochemical inertia. Thus, by using ammonium acetate buffer at pH 5.15, efficient separation with sensitive detection of MHPG, 5-HIAA, and HVA can be achieved (Fig. 1A). The applicability of this simple isocratic system is demonstrated by the analysis of CSF samples. The three major amine metabolites are clearly separated from other endogenous substances (Fig. 1B). The parent compounds noradrenaline, adrenaline, and dopamine, as well as 3,4-dihydroxyphenylacetic acid, were eluted before the compounds of interest and were well resolved from these metabolites. We could not obtain similar resolution with phosphate buffers. Solvent degassing is important in electrochemical detection. A reversed-phase system not requiring organic modifier is advantageous as on-line degassing leads to gradual evaporation of the organic modifier resulting in a change of mobile phase composition. The retention of the compounds can be simply controlled by pH and buffer concentration adjustments of the mobile phase without the need for inclusion of organic modifer. EDTA, included in the mobile phase to protect the electrode from metallic contamination, did not affect the retention or resolution of the amines. It can be omitted when an ultraviolet or a fluorescence detector is used.

Effect of pH on retention

The retention of the two acidic metabolites, 5-HIAA and HVA, was significantly affected by the pH of the mobile phase. At lower pH ionization of the acid groups was suppressed, and the molecules were therefore more strongly retained. MHPG is without an acid group and was thus virtually unaffected by pH changes.

Effect of buffer concentration on retention

Increasing the molar concentration of ammonium acetate decreased the

capacity ratios (k') of all three metabolites (Fig. 2). This is probably due to the more effective masking of residual silanol groups by NH_4^+ when higher concentrations of ammonium acetate were used. Although hydrophobic interaction is the main retention mechanism, absorption of solutes onto residual silanol sites may also operate to a lesser extent, judging from these retention behaviours. It is possible to use 0.5 *M* ammonium acetate as the eluent without causing problems to the detector, provided EDTA is present.

Optimum detection potential

The voltammograms of the three metabolites are shown in Fig. 3; whereas useful detection begins at +0.70 V for MHPG and HVA, 5-HIAA can be oxidised at lower potentials. An operating potential of +0.85 V was chosen for the simultaneous sensitive detection of all three compounds.

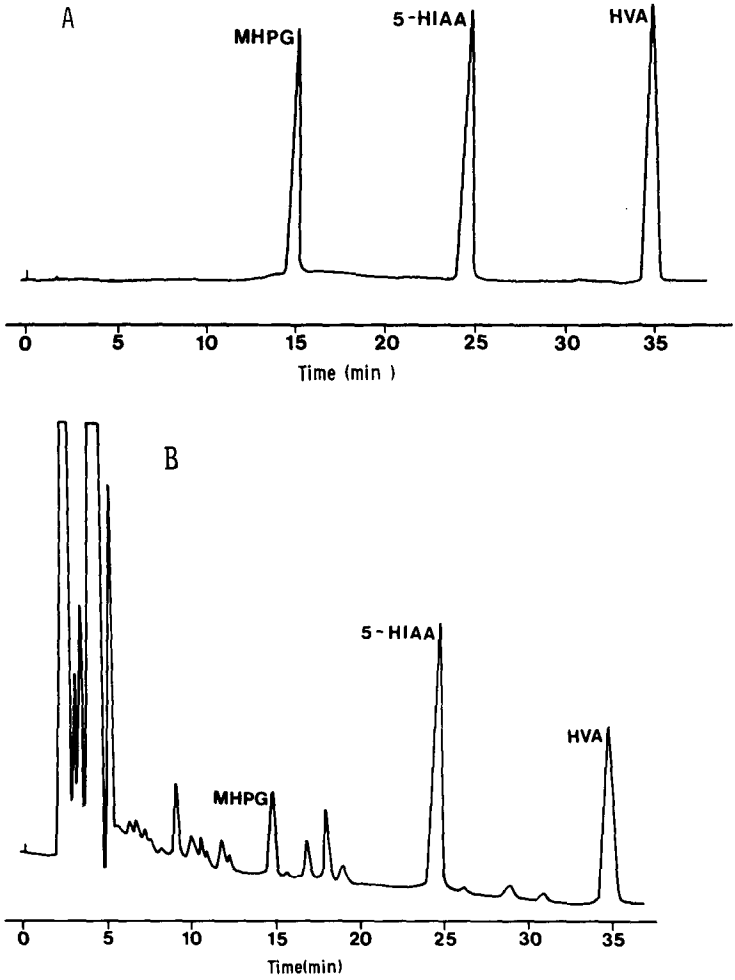

Fig. 1. Separation of MHPG, 5-HIAA and HVA in (A) standard solution of the compounds and (B) cerebrospinal fluid. Column: ODS Hypersil: mobile phase, 0.1 *M* ammonium acetate, pH 5.15.

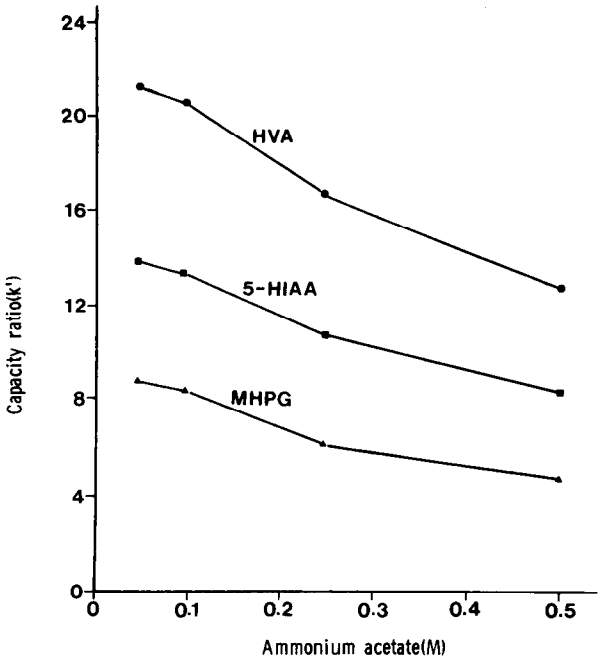

Fig. 2. Effect of molar concentration of ammonium acetate on the capacity ratios (k') of MHPG, 5-HIAA and HVA.

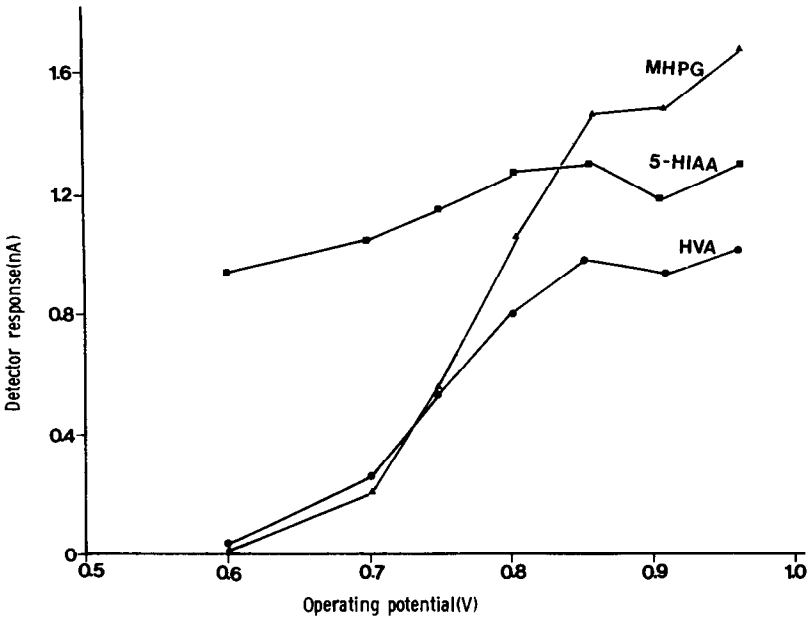

Fig. 3. Voltammograms for MHPG, 5-HIAA and HVA.

Reproducibility, linearity and recovery

The reproducibility of the method was tested by repeated injection (ten times) of a standard solution containing 50 nmol/l of each of the three metabolites. Standard deviations of ± 1% were achieved for peak response and k' values. The use of totally aqueous eluent which is unlikely to suffer from mobile phase composition changes during chromatography is largely responsible for the good reproducibility.

The standard curves were prepared by plotting the peak height against concentration in the range 5—35 nmol/l. Linear regression analysis data indicated no significant deviation from linearity (r = 0.9997). The intercept values also did not differ significantly from zero.

The recovery of a standard solution (50 nmol/l) from ultrafiltration through HPLC separation was also ± 1% rendering the use of an internal standard unnecessary.

The detection limit of MHPG, 5-HIAA and HVA were 30, 34 and 50 pg on column, respectively, based on a signal-to-noise ratio of 3.

MHPG, 5-HIAA and HVA levels in CSF

The catecholamine metabolite levels in CSF of depressed and neurological patients are shown in Table I. The results are well correlated with those previously reported [15]. No attempt was made to relate these levels with the clinical diagnosis of the patients although, as may be expected, there is a greater variation in the levels of 5-HIAA and HVA in the neurological cases than in the depressed patients.

TABLE I

AMINE METABOLITE LEVELS IN HUMAN CEREBROSPINAL FLUID

	MHPG (nmol/l)	5-HIAA (nmol/l)	HVA (nmol/l)
Depressed patients (n = 18)	50.9 ± 21.3	85.2 ± 34.5	162 ± 75
Neurological patients (n = 9)	51.0 ± 16.8	87.3 ± 64.9	293 ± 176

CONCLUSION

MHPG, 5-HIAA, and HVA in CSF can be separated isocratically from the parent compounds by reversed-phase chromatography with ammonium acetate solutions as eluents. The retention behaviour has been studied and it was concluded that effective resolution from all endogenous impurities required a mobile phase of 0.1 M ammonium acetate at pH 5.15.

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