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SIMULTANEOUS DETERMINATION OF 4-HYDROXY-3-METHOXY-PHENYLACETIC (HOMOVANILLIC) ACID AND OTHER MONOAMINE METABOLITES IN HUMAN LUMBAR CEREBROSPINAL FLUID

AN IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC STUDY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

We describe a direct analysis for the simultaneous quantitative determination of 4-hydroxy-3-methoxyphenylacetic (homovanillic) acid and other monoamine metabolites in human lumbar cerebrospinal fluid, utilizing reversed-phase high-performance liquid chromatography with amperometric detection. In addition, a rapid isocratic separation was developed for homovanillic acid in the presence of other endogenous compounds.

Twenty-five unselected diagnostic specimens of human lumbar cerebrospinal fluid were extracted with ethyl acetate and subsequently analyzed using the described method. Chromatographic peaks were identified on the basis of retention behavior and ratio of responses at several oxidation potentials.

Although our quantitative results correlate well with the literature values, the data were not interpreted clinically since samples were obtained from routine, diagnostic testing of patients admitted to the medical or neurologic services at the Mount Sinai Hospital.

INTRODUCTION

Since the biogenic amine hypothesis of affective disorders has been proposed [1, 2], the description and treatment of neurochemical abnormalities

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occurring in mania, depression, schizophrenia and Parkinson's disease, have centered on the measurement of monoamines in various physiologic samples [3—5]. The assessment of monoamine metabolism in the central nervous system is usually deduced from the metabolite levels of the parent brain amines in cerebrospinal fluid (CSF) [6] where findings are not obscured by peripheral contributions. 3,4-Dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxyphenylacetic (homovanillic) acid (HVA) are the major metabolites of dopamine (DA), while 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) and 4-hydroxy-3-methoxymandelic (vanillylmandelic) acid (VMA) are the central and peripheral catabolites of norepinephrine (NE).

Although specific assays for separate metabolites are available in the literature, there is a great need for a simple, integrated method for simultaneous measurement of several important metabolites in small volumes of physiological samples. The relatively low concentrations of monoamine metabolites in CSF have necessitated the use of sensitive analytical techniques such as gas chromatography—mass spectrometry [7—9], enzymatic—isotopic methods [10] and fluorimetric methods [11]. However, some of these techniques entail elaborate sample preparation, technical expertise and/or radiation hazards.

High-performance liquid chromatography (HPLC) coupled with amperometric detection has recently emerged as an excellent analytical tool for rapid and sensitive measurements of monoamine metabolites [12—17]. Thus, derivatization is not necessary and direct analyses are possible with minimal sample preparation. With the high-efficient microparticulate reversed-phase packings simultaneous determinations of a broad range of compounds of different polarities can be achieved in minimal time. In addition, aqueous buffers commonly employed in reversed-phase HPLC separation are functionally compatible with the amperometric detection.

Therefore, we have investigated the use of HPLC with amperometric detection for concurrent quantitative analysis of several monoamine metabolites along the NE and DA pathways. We also describe an isocratic elution mode for the assay of HVA when only this metabolite is of clinical interest. The determination of HVA in CSF is of great importance particularly in Parkinson's disease and affective disorders which involve impairment of central dopaminergic tracts. These methods are applied to the assay of monoamine metabolites in samples of human lumbar CSF from patients admitted to the medical or neurologic services at the Mount Sinai Hospital. The described analytical procedure holds promise for routine clinical investigations of neurochemical substances.

EXPERIMENTAL

Materials and methods

A Model 6000A solvent delivery system, Model U6K universal injector and Model 660 solvent programmer, all from Waters Assoc. (Milford, MA, U.S.A.) were used in the course of this study. Chromatographic effluents were monitored using a voltametric/amperometric detector, Model E 611, with an EA 1096 detector cell (Brinkmann Instruments, Westbury, NY, U.S.A.). This

detector operates on a three-electrode potentiostatic principle and employs glassy carbon working and auxiliary electrodes and a Ag/AgCl reference electrode. The detector employs a wall-jet design and the effective cell volume is approximately 1 μl. The chromatographic column was an Ultrasphere ODS (5 μm average particle diameter, 250 mm × 4.6 mm I.D.) from Altex Scientific (Berkeley, CA, U.S.A.).

All reference compounds, purchased from Sigma (St. Louis, MO, U.S.A.), were of the highest purity (ACS certified). Potassium dihydrogen phosphate was purchased from Mallinckrodt (St. Louis, MO, U.S.A.), methanol (distilled-in-glass) from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) and ethyl acetate (pesticide grade) from Fisher Scientific (Springfield, NJ, U.S.A.). Solutions of reference compounds were prepared in distilled-deionized water and were kept refrigerated when not in use.

Chromatographic conditions

For the determination of several monoamine metabolites, a gradient elution mode of reversed-phase HPLC was used. The low-strength eluent was a solution of 0.1 *M* KH_2PO_4 (pH 2.50) and the high-strength eluent was a mixture of anhydrous methanol and distilled-deionized water (3 : 2, v/v). The low-strength eluent was always filtered through a Millipore membrane filter (Millipore, Bedford, MA, U.S.A.), type HA, pore size 0.22 μm. Both the low- and high-strength eluents were purged with high-purity helium in order to expel the dissolved air. A 35-min linear gradient from 0 to 100% of the high-strength eluent was used. Although the ionic strength was zero at the upper limit of the gradient, the last compound of interest, HVA, was eluted at 77% of the gradient course.

In addition, an isocratic elution mode was developed for the analysis of HVA. The eluent was a mixture of 25% methanol in 0.08 *M* KH_2PO_4 (pH 2.50).

The flow-rate was 1.2 ml/min both in the gradient and isocratic elution. The temperature was ambient and the peaks in chromatographic effluents were detected amperometrically at an oxidation potential of +0.700 V vs. Ag/AgCl electrode.

Samples

Routine unselected specimens of human lumbar CSF were obtained from the patients admitted to the neurologic services at the Mount Sinai Hospital. Prior to analysis, samples were stored frozen at −20°C. Since CSF contains many oxidizable compounds, the selectivity of the amperometric detection is usually low at oxidation potentials optimized for maximum sensitivity of the compounds of interest. This problem is overcome by means of an ethyl acetate extraction, which is specific for the phenolic acids. Therefore, CSF samples were acidified with 6 *M* hydrochloric acid (to pH less than 1) and extracted twice with 3-ml portions of ethyl acetate and once with a 6-ml portion. The ethyl acetate extracts were pooled, evaporated to dryness under a stream of dry nitrogen and reconstituted with water to a volume smaller than the original CSF sample. Thus it was possible to analyze extracts of as little as 0.200 ml of CSF. The extracts were immediately frozen.

Extraction efficiency

Since the CSF samples were unselected, their composition varied considerably, depending upon the patient history. Thus it was impossible to find an internal standard which would be structurally related to the compounds of interest and yet would elute free from interferences from the endogenous compounds. For this reason, the efficiency of the extraction procedure was determined using external standards which were carried through the sample preparation procedure under identical conditions. The concentrations of the standards were of the same order of magnitude as their endogenous levels in the CSF samples. The percent recoveries of the compounds of interest were between 94.5 and 98.0%. The coefficient of variation was 0.15% ($n = 4$).

Peak identification

Initial peak identification of the compounds of interest was performed on the basis of liquid chromatographic retention behavior and co-injection with the reference compounds.

The electrochemical oxidation potential, optimized for the analysis of monoamine metabolites, and the specificity of the extraction procedure for the phenolic compounds were selective enough to be a confirmatory test when used in combination with other methods.

In addition, ratios of responses at several oxidation potentials (+0.600 V to +1.000 V) were computed for the reference compounds and compared with those of the peaks in CSF extracts. These ratios were proved to be useful in the analysis of biological mixtures detected amperometrically [17].

Linearity of detector response and limits of detection

Detector responses were found to increase linearly with concentration in the range between 100 pg and 600 ng. The average detection limit for the compounds of interest was approximately 40 pg with a 20% rise in the baseline under the chromatographic conditions used.

Quantitative analysis

Chromatographic peaks were quantified on the basis of peak areas and comparison of the reference compound response. Peak areas were computed manually using the width-at-half-height method. Triplicate injections of the same sample gave coefficients of variation of peak area and retention times of 1.0 and 0.5%, respectively.

Precision

Precision of the analytical procedure was determined by chromatographing aliquots of CSF samples. The within-day precision of quantitative results gave a coefficient of variation of approximately 0.7% ($n = 4$) for all compounds under study; the day-to-day precision measurements produced coefficients of variation of approximately 1.1% ($n = 4$).

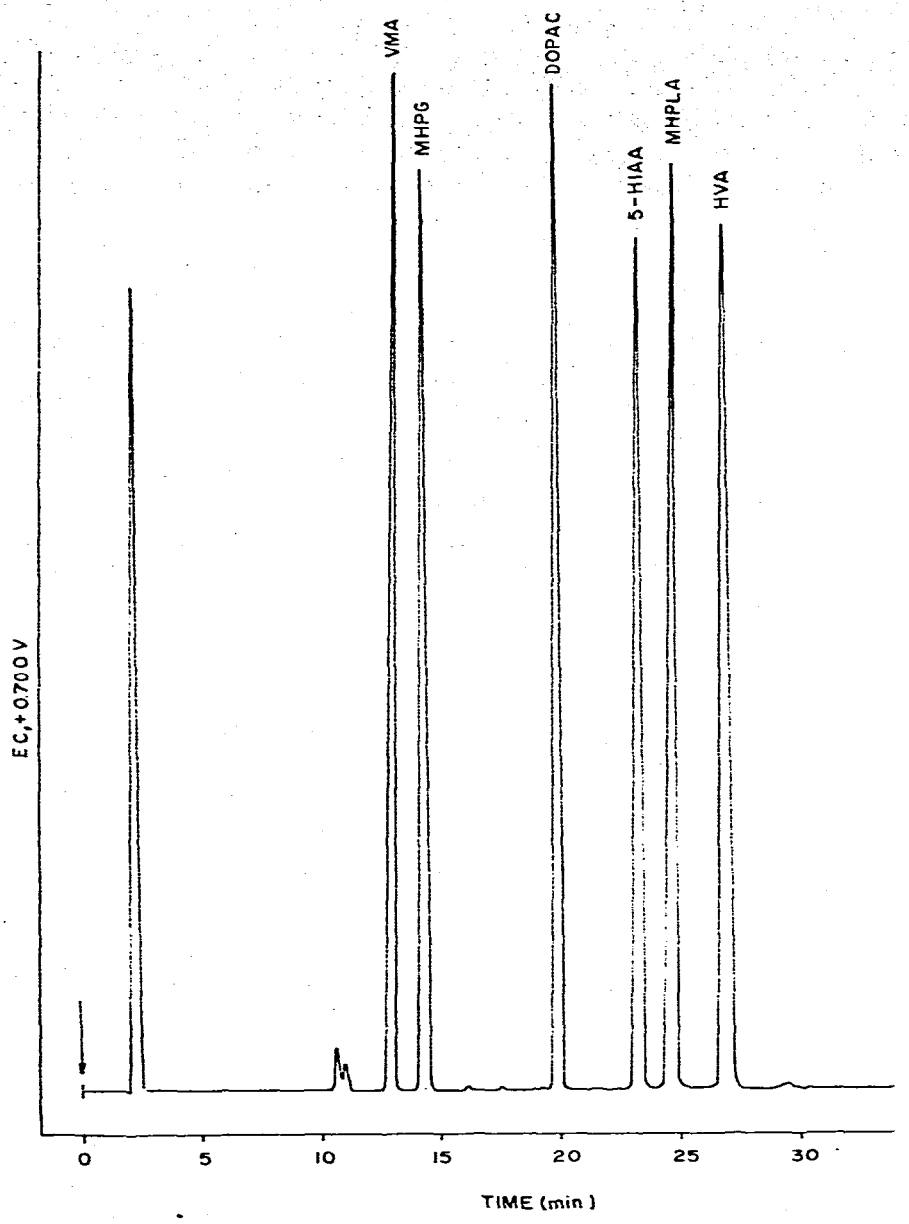

Fig. 1. Separation of a synthetic mixture of reference compounds. Peak identity and quantity: 3-methoxy-4-hydroxymandelic acid (VMA, 19.2 ng), 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG, 32 ng), 3,4-dihydroxyphenylacetic acid (DOPAC, 35.0 ng), 5-hydroxyindole-3-acetic acid (5-HIAA, 10.2 ng), 3-methoxy-4-hydroxyphenyllactic acid (MHPLA, 70.0 ng), 4-hydroxy-3-methoxyphenylacetic acid (HVA, 30.0 ng). Chromatographic conditions: column, Ultrasphere ODS (5 μm average particle size); eluents, low-strength: 0.10 *M* KH_2PO_4, pH 2.50; high-strength, methanol—water (3 : 2, v/v); gradient, linear from 0 to 100% of the high-strength eluent in 35 min; flow-rate, 1.2 ml/min; temperature, ambient; detection, amperometric at +0.700 V vs. Ag/AgCl; sensitivity, 75 nA full scale.

Interferences

No interferences were observed in the course of this study. It is to be expected, however, that in chromatograms of CSF samples from patients undergoing different medication treatments, other electroactive compounds may be found, although it is difficult to estimate the extent of this effect, we believe that slight modifications in the gradient slope would afford adequate resolution.

RESULTS AND DISCUSSION

Since the measurement of the major monoamine metabolites in human lumbar CSF provides useful information regarding the role of neurotransmitters in several neuropsychiatric disorders, there is considerable interest in rapid and reliable methods which can detect these compounds at their endogenous levels. The ability of HPLC coupled with amperometric detection to analyze and monitor sensitively oxidizable catechol and indole compounds is well documented in the literature. However, most work in this area deals either with specific assays for a single monoamine metabolite or concurrent determinations of several metabolites using the ion-association (ion-pairing) technique.

Therefore, we have developed a direct, integrated assay for several major metabolites of NE and DA. The reversed-phase HPLC system allows baseline separation of VMA, MHPG, 5-hydroxyindole-3-acetic acid (5-HIAA), 3-methoxy-4-hydroxyphenyllactic acid (MHPLA) and HVA. Fig. 1 shows a typical chromatogram of a synthetic mixture of the acidic metabolites. Prior to the analysis a study was conducted to determine the optimal oxidation potential to ensure adequate sensitivity while still maintaining the desired selectivity. From the long-term studies (1—4 months without resurfacing of the glassy carbon electrodes) it was concluded that the potential of +0.700 V was the best compromise between detection sensitivity and electrode stability. Therefore, successive analyses of the CSF samples were performed at that potential.

The efficiency of the ethyl acetate extraction was determined for each compound of interest and was found to be quantitative (> 90%). The specimens were extracted as described and subsequently chromatographed. The ethyl acetate extracts were evaporated to dryness under a stream of nitrogen and the residues were reconstituted with water to a volume smaller than the original CSF specimen. Thus, samples were pre-concentrated (approximately 2—4 times) and a complete analysis and identification of the compounds of interest was achieved using a maximum volume of 200 μl of CSF. Fig. 2 illustrates a chromatogram of the ethyl acetate extract of CSF. HVA occurs at levels several orders of magnitude higher than those of the other metabolites and therefore lower detector sensitivities were used. Since unselected diagnostic CSF samples were analyzed, the profiles of the constituent compounds varied considerably. A chromatogram of the ethyl acetate extract of another CSF sample is shown in Fig. 3.

Since some diseases involve derangements in only one neurotransmitter, it is important to be able to monitor one specific metabolite rapidly and routinely. For example, HVA, a major end-product of DA metabolism, is of

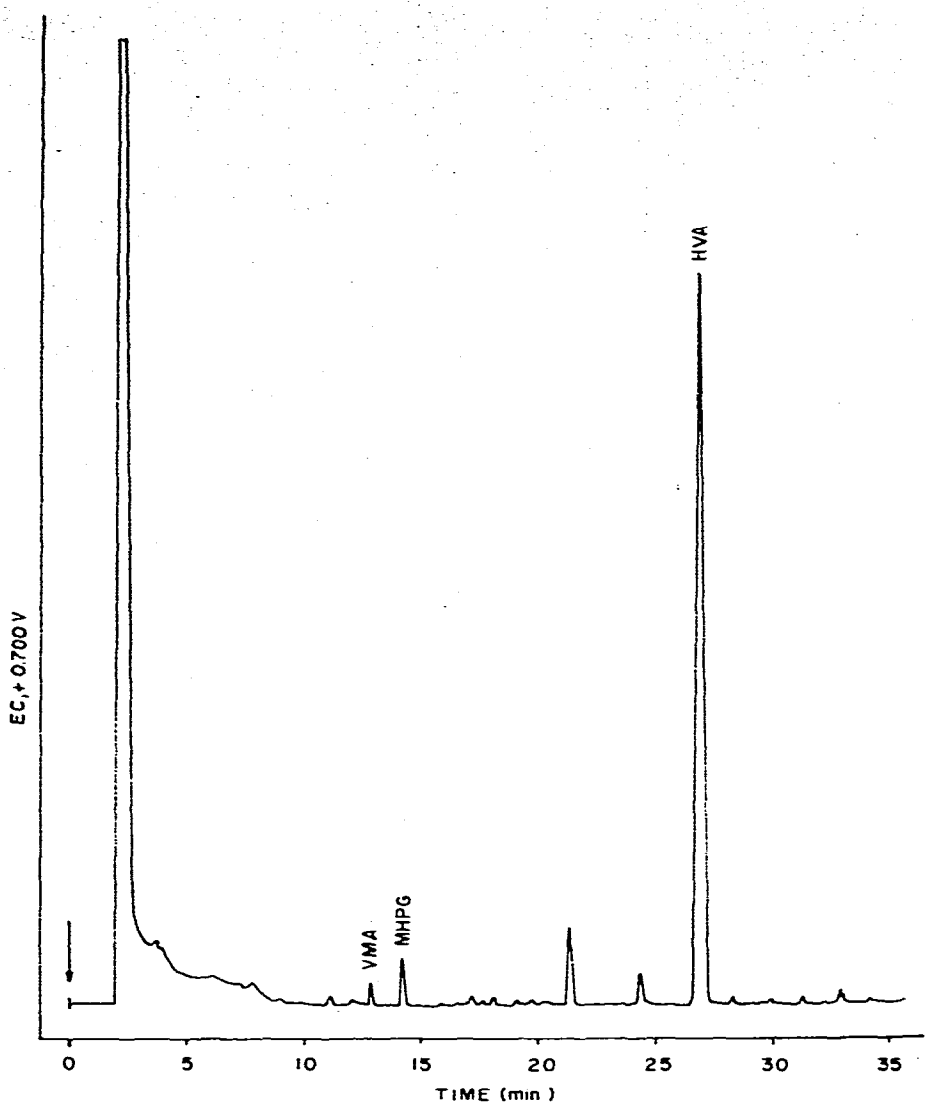

Fig. 2. Chromatogram of the ethyl acetate extract of a sample of human lumbar CSF. Chromatographic conditions as in Fig. 1. Volume of the extract injected: 60 μl (180 μl of CSF).

critical importance in Parkinson's disease. Therefore, an isocratic elution mode of reversed-phase HPLC was developed for the determination of HVA alone. Fig. 4 illustrates the separation of a synthetic mixture of monoamine metabolites under isocratic conditions. HVA is eluted rapidly and is free from interferences from other electroactive compounds. The developed analytical procedure was applied to the analysis of HVA in a CSF sample as shown in Fig. 5. In order to ensure that the chromatographic band of HVA did not contain any unresolved impurities, the peak was quantified both under the gradient elution and isocratic conditions. The results were identical. In addition, ratios of responses at several selected potentials were computed for the HVA refer-

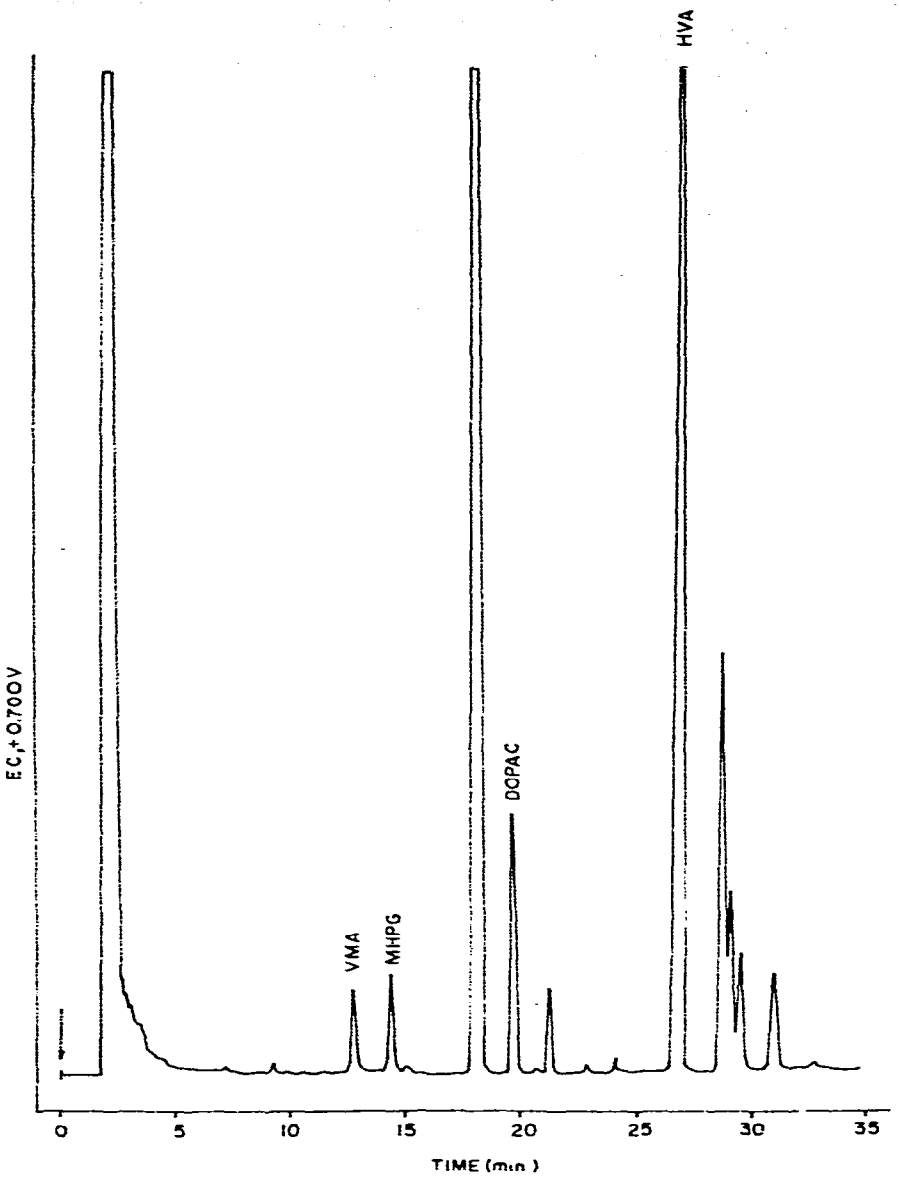

Fig. 3. Chromatogram of the ethyl acetate extract of a sample of human lumbar CSF. Chromatographic conditions as in Fig. 1. Volume of the extract injected: 60 µl (190 µl of CSF).

ence solution and the peak in the extract. In several CSF samples, an unidentified peak was found to elute immediately prior to HVA. However, since it was 90% resolved from the HVA peak, it did not interfere with the quantification of HVA.

The results of the quantitative analysis of VMA, MHPG, DOPAC, and HVA in 25 samples of human lumbar CSF are given in Table I. Since diagnostic CSF specimens were used, it is difficult to interpret the quantitative data and correlate it with medical and/or neurologic diagnosis. Furthermore, no

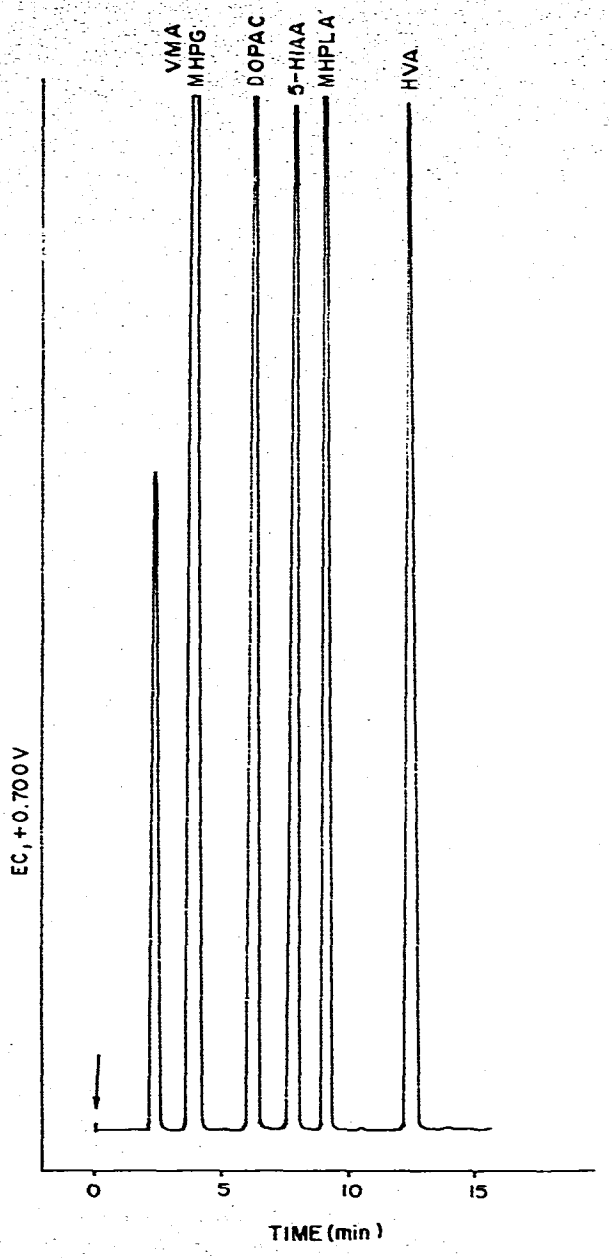

Fig. 4. Chromatogram of synthetic mixture of reference compounds analyzed under isocratic conditions. Peak identity and quantity as in Fig. 1. Chromatographic conditions: column, Ultrasphere ODS (5 μm average particle size); eluent, 25% methanol in 0.08 *M* KH_2PO_4, pH 2.50; flow-rate, 1.2 ml/min; temperature, ambient; detection, amperometric at +0.700 V; detector sensitivity, 75 nA full scale.

effort was made to restrict medications. Despite such problems, the concentrations are in the range of those previously reported [7, 9].

The primary objective of this report was to describe the analytical procedure for the quantitative determination of the VMA, MHPG, DOPAC, and

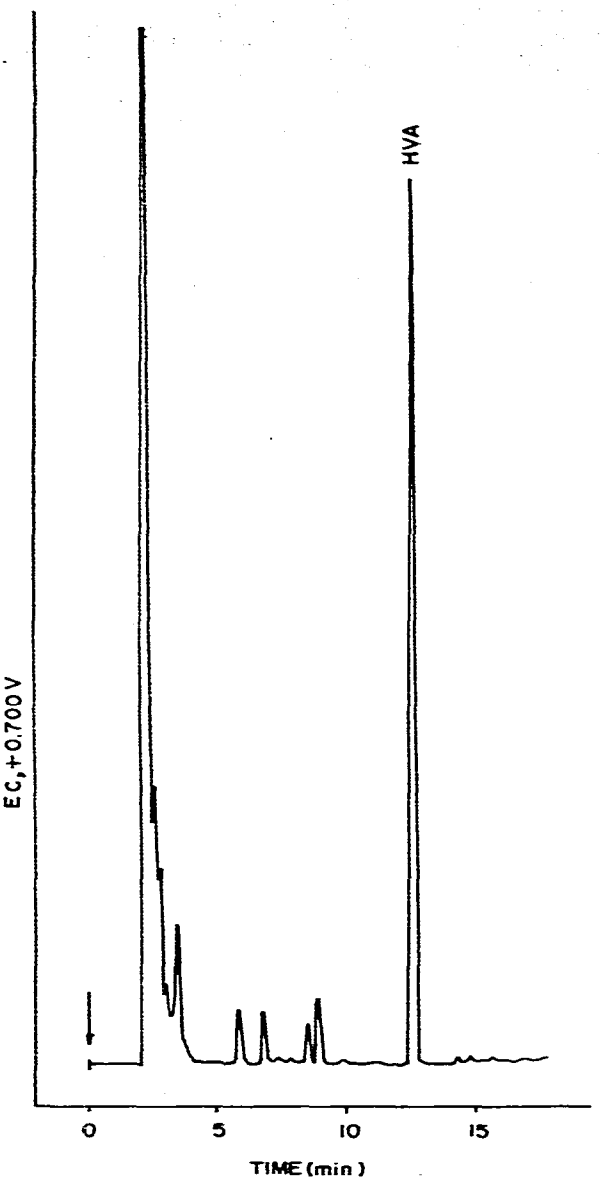

Fig. 5. Chromatogram of the sample shown in Fig. 2, analyzed under isocratic conditions. Chromatographic conditions as in Fig. 4. Volume of the extract injected, 60 μl (180 μl of CSF).

HVA in CSF. Because of its simplicity and rapid acquisition of data, we believe the technique will be a useful tool for routine assessment of CSF profiles of monoamine metabolites in small volumes of samples.

TABLE I

CONCENTRATIONS OF SOME MONOAMINE METABOLITES IN 25 SAMPLES OF HUMAN LUMBAR CSF

Compound	Range (ng/ml of CSF)
VMA	0.300— 0.66
MHPG	0.850—17.61
DOPAC	0.280— 0.790
HVA	10.310—30.47

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