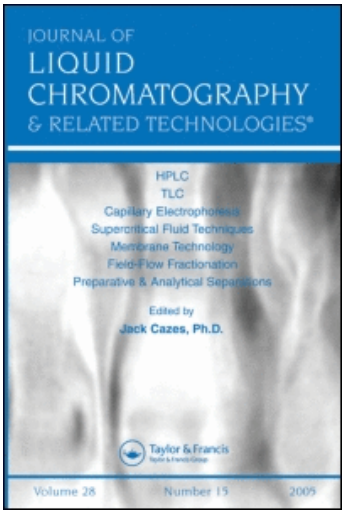

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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L. Elrod Jr.[a]; G. L. Mayer[a]
[a] Bioanalytical Systems, Inc, West Lafayette, Indiana

To cite this Article Elrod Jr., L. and Mayer, G. L.(1985) 'Determination of Monamine Metabolites in Human Cerebrospinal Fluid by High Performance Liquid Chromatography with Electrochemical Detection', Journal of Liquid Chromatography & Related Technologies, 8: 10, 1817 — 1827
To link to this Article: DOI: 10.1080/01483918508074097
URL: http://dx.doi.org/10.1080/01483918508074097

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(10), 1817-1827 (1985)

DETERMINATION OF MONAMINE METABOLITES IN HUMAN CEREBROSPINAL FLUID BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

L. Elrod, Jr. and G. L. Mayer
Bioanalytical Systems, Inc,
2701 Kent Avenue
West Lafayette, Indiana 47906

ABSTRACT

An optimized high performance liquid chromatographic (HPLC) separation was developed to determine the monoamine metabolites 3-methoxy-4-hydroxyphenyl glycol (MHPG), 4-hydroxy-3-methoxyphenylacetic acid (HVA) and 5-hydroxyindole-3-acetic acid (5-HIAA) in human cerebrospinal fluid (CSF). After deproteinization with trichloroacetic acid (TCA), CSF samples were chromatographed without further prepurification. Chromatographic separations were achieved isocratically using a reverse phase Biophase ODS (5 μm) analytical column and an eluent containing 0.05 M ammonium acetate at pH 5.3 and 0.9% tetrahydrofuran. An amperometric electrochemical detector with a thin-layer glassy carbon working electrode was used. The working electrode was operated at +0.80 V versus a Ag/AgCl reference. Quantitation was performed by comparing peak heights of the subject compounds obtained from the sample preparation to those of a standard treated identically. Recovery of the metabolites ranged from 71.1 to 110% when added to CSF in concentrations of 2.0 to 63 ng/ml. Assay precision (relative standard deviations) for pooled CSF ranged from ±5.7 to ±9.9% and the monoamines were detectable (S/N ≳ 3) at 20 to 40 pg injected in standards.

 0148-3919/85/0810-1817$3.50/0

INTRODUCTION

The monoamines 3-methoxy-4-hydroxyphenyl glycol (MHPG), 4-hydroxy-3-methoxyphenylacetic acid (HVA) and 5-hydroxyindole-3-acetic acid (5-HIAA) are major metabolites of norepinephrine (NE), dopamine (DA) and serotonin (5-HT) respectively. The concentrations of these metabolites in cerebrospinal fluid (CSF) reflect neuronal activity in the central nervous system. Increasing interest is being shown in determining monoamine metabolites since abnormal levels have been associated with a variety of psychiatric (1-2) and neurological disorders (3-5). The combined sensitivity and specificity afforded by high performance liquid chromatography with electrochemical detection (LCEC) allow the determination of these compounds at the low ng/ml levels found endogenously in CSF.

Recent accounts in the literature report the determination of monoamine metabolites in CSF using LCEC after ethyl acetate extraction (6) or by direct injection (7-12). The direct injection approach allows a very simple and rapid sample preparation while minimizing analyte losses. While published methods for the metabolites resolve the three compounds of interest to varying degrees, few show the presence of 5-HT and tryptophan (TRP) in CSF sample preparations. These constitutents, present in high concentrations (relative to the monoamine metabolites) are possibly adsorbed strongly onto reverse phase packing and elute at prohibitively long retention times. This procedure describes an optimized isocratic chromatographic system to separate the monoamine metabolites from other endogenous compounds in CSF while eluting TRP, 5-HT and more strongly retained components in less than 40 minutes. A reliable measurement is achieved using only 100 µL of human CSF.

MATERIALS AND METHODS

Reagents

Ammonium acetate and 70% perchloric acid were reagent grade and were obtained from Fisher Scientific Co. (Fair Lawn, NJ).

Tetrahydrofuran (THF) was HPLC grade from Burdick and Jackson Labs (Muskegon, MI). Acetic acid and trichloroacetic acid (TCA) were reagent grade from Mallinckrodt Chemical (Paris, KY). Disodium ethylenediaminetetraacetic acid (Na_2EDTA) and L-cysteine were reagent grade from Eastman Kodak Co. (Rochester, NY) and Sigma Chemical Co. (St. Louis, MO) respectively.

Solvents for standard preparations contained 0.1 M perchloric acid and 0.1 M perchloric acid, 0.1% (W/V) cysteine in deionized water. A solution containing 10% TCA and 0.1% cysteine (W/V) was prepared in deionized water for sample deproteinization.

Liquid Chromatograph and Conditions

The chromatographic system consisted of a BAS Model LC-154 T chromatograph equipped with a Model LC-4B/17 electrochemical detector and a Model TL-5 glassy carbon working electrode (all from Bioanalytical Systems, Inc., West Lafayette, IN). The working electrode was operated at +0.80 V versus a Ag/AgCl reference electrode. Detector sensitivity was 10 nA for 1 volt full scale deflection.

Chromatographic separations were achieved using a 25 cm x 4.6 mm Biophase ODS (5 µm) analytical column (Bioanalytical Systems, Inc., West Lafayette, IN). The HPLC eluent was a mixture of 9 ml of THF and 1 liter of buffer containing 0.05 M ammonium acetate and 0.1 mM Na_2EDTA which had been adjusted to pH 5.3 with acetic acid. Prior to use, the solution was filtered through a 0.2 µm nylon membrane (Rainin Instrument Co., Woburn, MA) and degassed under vacuum with stirring. Injections of 20 µL were made with the system's fixed loop injection valve. A flow rate of 1.5 ml/min was maintained at 2400 psi back pressure.

CSF Samples

CSF samples collected by lumbar puncture for other diagnostic purposes were obtained from Chicago, IL area hospitals. All samples were stored frozen until processed.

Standard Preparations

Standards of HVA, 5-HIAA and MHPG (obtained as the piperazine salt) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Stock solutions of each monoamine at concentrations of 200 μg/ml were prepared in 0.1 M $HClO_4$, 0.1% cysteine. A mixed working standard containing 10 ng/ml of MHPG and 50 ng/ml each of HVA and 5-HIAA was prepared by combining and diluting the stock standards in 0.1 M $HClO_4$.

Assay Procedure

Aliquots of 100 μL of the monoamine standard solution were pipeted into 1.5 ml disposable centrifuge tubes. To each tube 20 μL of 10% TCA, 0.1% cysteine solution was added. Replicate standard preparations were typically prepared and stored in the freezer until injected.

CSF samples were stored frozen until immediately before use to minimize losses of the metabolites. Samples were processed one at a time as described for the standards. After addition of the deproteinization solution, the samples were placed in an ice bath for ten minutes and centrifuged for five minutes. The clear solution was immediately injected.

The monoamine metabolite concentrations were calculated as follows:

$$\text{Monoamine Conc.}_{(\text{Spl.})} = \frac{\text{Peak Height (Spl.)}}{\text{Peak Height (Std.)}} \times \text{Std. Conc. (ng/ml)}$$

RESULTS AND DISCUSSION

In order to determine the required operating potential to simultaneously quantitate each of the monoamines, hydrodynamic voltammograms were generated by repetitively chromatographing 2 ng of each metabolite. An applied potential of +0.80 V was chosen for optimum detection of all metabolites.

Chromatograms of a standard preparation and CSF sample preparation obtained as described in the procedure are presented in

Figure 1. The chromatographic conditions described in the text were used. Also presented in the Figure is a chromatogram of a synthetic standard mixture containing the metabolites in combination with DOPAC, 5-HT and TRP (also present in CSF). As shown, the monoamine metabolites elute as well-resolved peaks within 12 minutes. Since 5-HT, TRP and two unidentified late eluting compounds are present in CSF, a run time of about 38 minutes is required for samples. By using TCA to precipitate the proteins from the CSF samples, the early portion of the chromatogram was much clearer than when perchloric acid was used for the same purpose. Less sample dilution is also required by using this reagent since it is very efficient at protein precipitation.

The chromatographic conditions described in the text have been optimized for the determination of the monoamine metabolites in CSF using the Biophase ODS 5 μm column. The amount of retention which the components of interest have on the stationary phase is controlled by the type of buffer, solution pH and organic modifier used. HPLC eluents based on acetate, phosphate and citrate/ perchlorate buffers at pH 5.1-5.3 gave slightly less resolution for the peaks of interest in CSF sample preparations than the ammonium acetate system described. When methanol or acetonitrile were used as organic modifiers, less acceptable resolution also resulted, as shown in Figure 2. The conditions used in Figure 2 have been adjusted slightly to obtain a capacity factor for MHPG of approximately 4. This gives a clear comparison of the effect which the modifier has on the separation.

The effect of the mobile phase pH on the HPLC separation for monoamine metabolites is demonstrated in Figure 3. Here capacity factors for monoamines and other components found in CSF are plotted versus eluent pH values. The data were generated by chromatographing standard solutions using a 0.08 M phosphate mobile phase at pH 4.9 to 5.7. As shown in the figure, the pH change of the mobile phase has no effect on the retention of the neutral component MHPG. However, capacity factors for the acidic components 5-HIAA and HVA more than double by decreasing the

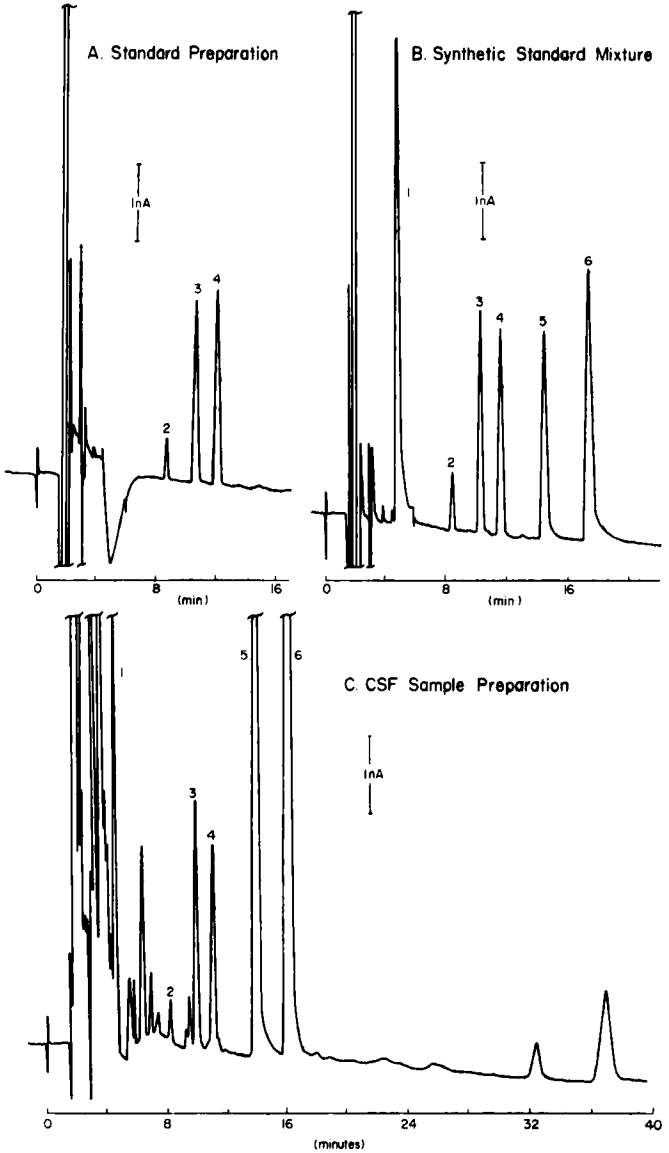

FIGURE 1. Chromatograms of Standard preparations (A,B) and CSF sample preparation (C) using conditions stated in text. Solutions A and C were prepared according to the assay procedure described in text, and solution (B) is a synthetic standard mixture prepared in 0.10 $HClO_4$. Peak identities: 1 = DOPAC, 2 = MHPG, 3 = HVA, 4 = 5-HIAA, 5 = TRP and 6 = 5-HT.

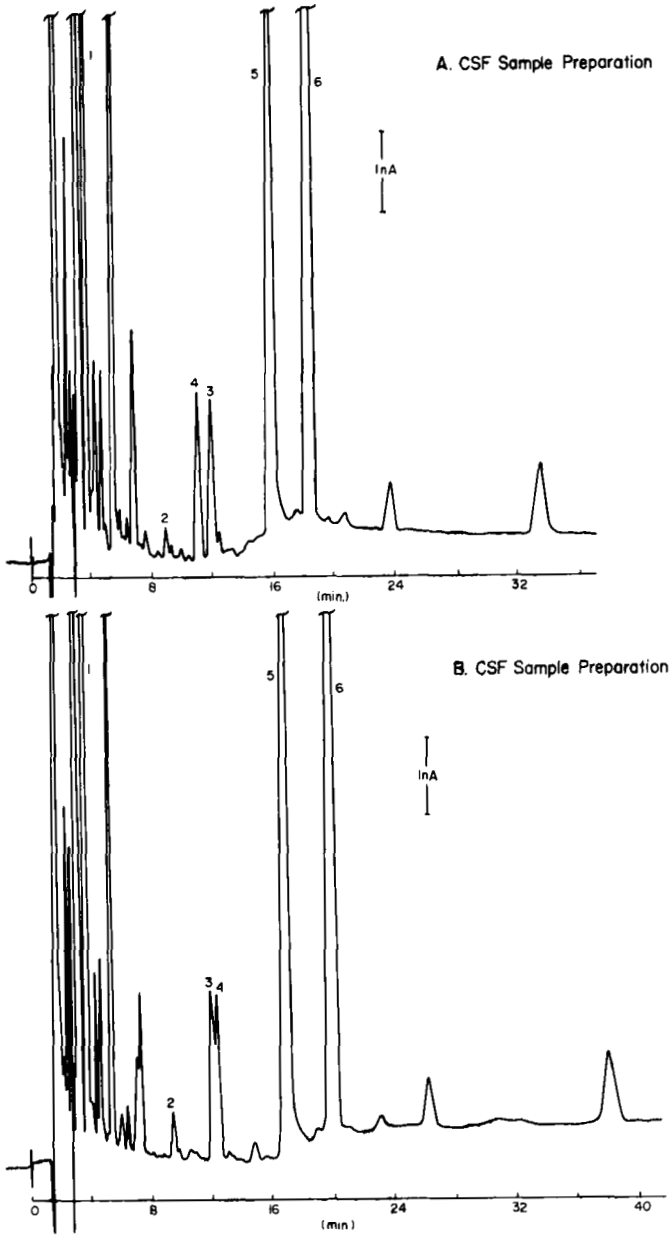

FIGURE 2. Chromatograms for CSF samples using various organic modifiers. Samples prepared as described in text; peak identities same as in Figure 1. Mobile Phase A = [0.05 M NH_4OAC, 0.1 mM EDTA @ pH 5.3]: methanol (1000:40). Mobile Phase B = [0.05 M NH_4OAC, 0.1 mM EDTA @ pH 5.3]: acetonitrile (1000:15).

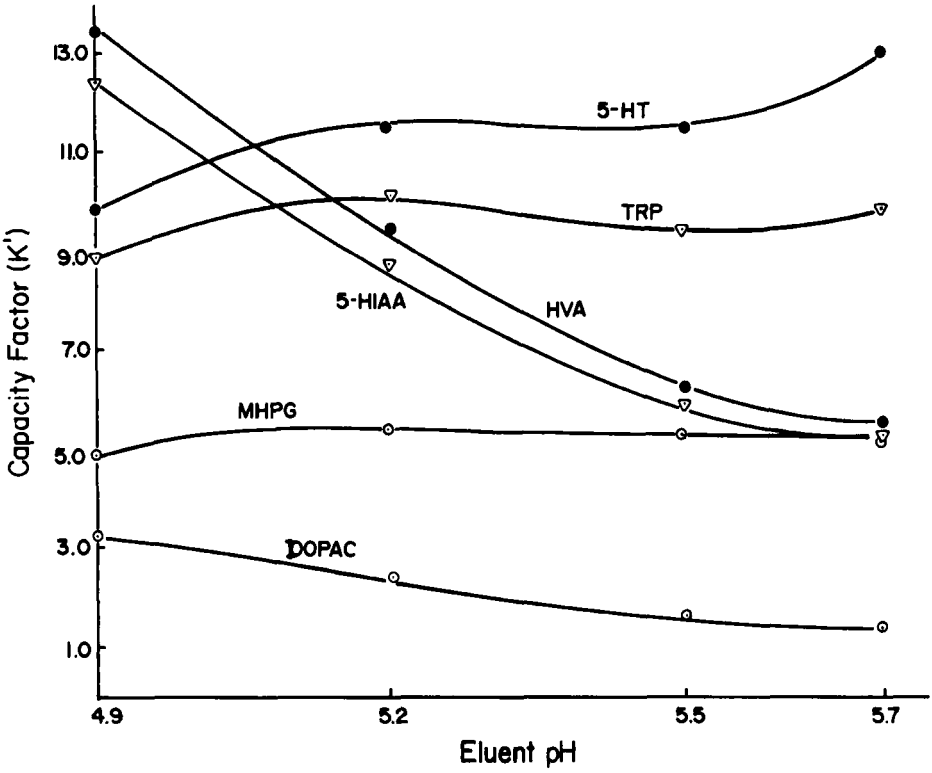

FIGURE 3. Capacity factors (K') plotted versus eluent pH for components found endogenously in CSF. A 0.08 M phosphate buffer (no organic modifier) was used as the eluent.

eluent pH from 5.7 to 4.9. This trend results from suppressing the ionization of the acids at the lower pH values. By adjusting the eluent pH from 5.2 to 5.4, 5-HIAA and HVA are resolved from MHPG, but still elute before the large TRP peak present in CSF.

Linearity of the detector response for monoamines was demonstrated by chromatographing standard solutions containing 8.4 to 70 ng/ml of the components of interest. As shown in Figure 4, plots of detector response (nA) versus concentration (ng/ml) were linear (correlation coefficients 0.9998- 0.9999) and essentially

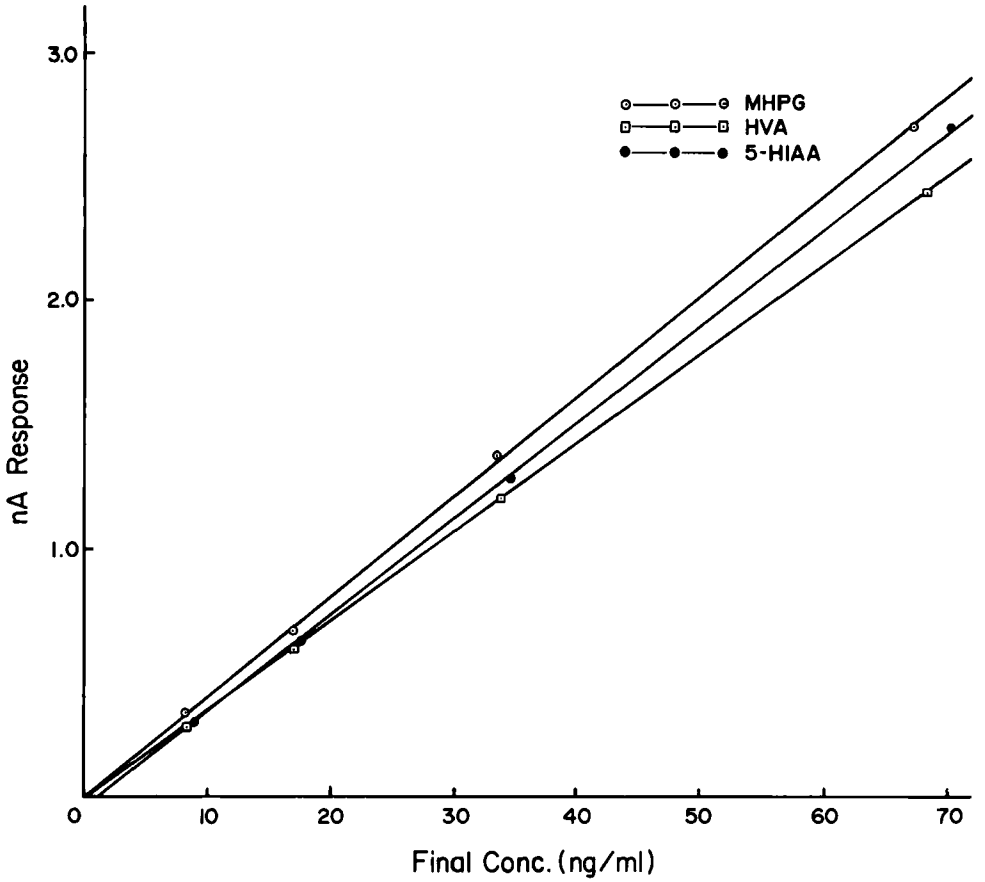

FIGURE 4. Linearity Curves for monoamine metabolites. Standard solutions were treated as described in the assay procedure. Final concentrations are plotted versus peak current response using 20 µL injections.

intersected the origin. Using the chromatographic conditions shown in text, 20 to 40 pg of monoamines injected are detectable (S/N $\approx$ 3) in standards.

To determine recovery of monoamine metabolites from CSF, 100 µL aliquots of a CSF pool were spiked with 5 µL of a standard mixture containing 0.20 to 6.33 ng of each component. The sam-

TABLE 1
Standard Addition and Recovery Data of
Monoamine Metabolites in CSF

MHPG		HVA		5-HIAA	
ng Added*	%Recovery**	ng Added*	%Recovery**	ng Added*	%Recovery**
0.20	100	1.02	80.4	1.06	107
0.40	82.5	2.04	71.1	2.11	110
0.81	90.1	4.08	74.3	4.22	107
1.21	84.3	6.13	74.2	6.33	107

*ng of monoamine added to 100 µL of pooled CSF.
**Corrected for endogenous monoamine content.

TABLE 2
Precision Data for Monoamine Metabolite Assay in CSF
Concentrations ng/ml for pooled CSF

	MHPG	HVA	5-HIAA
N	11	11	11
Mean	8.5	57.8	51.6
Range	7.7-9.9	54.3-63.1	47.4-54.6
RSD	±9.9%	±5.7%	±6.4%

ples were assayed as described in the procedure and the recoveries were calculated correcting for the amounts of metabolites present endogenously. Recovery data are shown in Table 1. As shown, recoveries ranged from 84.3-100% for MHPG, 71.1-80.4% for HVA and 107-110% for 5-HIAA.

Assay precision was determined by quantitating the monoamine metabolites in a CSF pool according to the procedure. Precision data were collected by two analysts over three different days and are summarized in Table 2. As shown relative standard deviations

for MHPG, HVA and 5-HIAA were ±9.9%, ±5.7% and ±6.4% respectively.

ACKNOWLEDGEMENT

The authors thank Ms. Katie Merriman for her assistance in the preparation of the manuscript.

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