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Short communication

Simultaneous determination of tryptophan, 5-hydroxytryptophan, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, 4-hydroxy-3-methoxyphenylacetic acid and 3-methoxy-4-hydroxyphenylglycol in human cerebrospinal fluid

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Abstract

Serotonin (5-hydroxytryptamine) metabolism may be influenced by its precursor tryptophan. A method utilizing reversed-phase high-performance liquid chromatography and electrochemical and ultraviolet detection with a mobile phase composed of acetate buffer and methanol has been developed for determination of tryptophan, its metabolites 5-hydroxytryptophan, serotonin, 5-hydroxyindoleacetic acid, as well as 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) and 3-methoxy-4-hydroxyphenylglycol in human cerebrospinal fluid (CSF). The electrochemical potential is set at 0.6 V in order to reduce the background current. Since tryptophan is not electroactive at this potential, it is detected by ultraviolet absorbance. The present method is simple, rapid, specific and accurate as compared with a previously reported method. No sample pretreatment is necessary and it takes ca. 20 min to run a sample. The concentrations of the compounds measured in CSF are similar to those obtained by HPLC in previous reports, although there are still arguments about the true level of serotonin in CSF.

1. Introduction

It is generally considered that the measurement of monoamine metabolites can provide insight into the rate of release or turn-over of their parent amines in the brain. Many HPLC methods have been developed for the measurement of monoamine metabolites with or without their parent amines and/or precursors in different combinations either in cerebrospinal fluid (CSF) or other biological samples [1–3]. For CSF samples, there are methods to measure 3-methoxy-4-hydroxyphenylglycol (MHPG), 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA) and 5-hydroxyindoleacetic acid (5-HIAA), the respective main metabolites of norepinephrine (NE), dopamine (DA) and 5-hydroxytryptamine (5-HT, or serotonin) [4], or 5-HT and 5-HIAA [3], or tryptophan, tyrosine, 5-HIAA and HVA [5], or HVA, MHPG, 3-methoxy-4-hydroxymandelic acid (VMA) and 3,4-dihydroxyphenylacetic acid (DOPAC) [6], or

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tryptophan, 5-hydroxytryptophan (5-HTP), 5-HT, 5-HIAA, HVA, 5-hydroxytryptophol and indoleacetic acid [7]. Because of the existence of interreactions between the DA, NE and 5-HT systems and the influence of precursors on the serotonin metabolism, it is necessary to develop a method to measure the main monoamine metabolites as well as the compounds related to the serotonin pathway.

We report here a simple, rapid, specific and accurate reversed-phase HPLC method with electrochemical (ED) and ultraviolet (UV) absorbance detection using a mobile phase of acetate buffer and methanol for the simultaneous determination of HVA, MHPG, 5-HIAA, tryptophan, 5-HTP and 5-HT in CSF, which allows to detect the three principal monoamine metabolites as well as the four compounds related to the serotonin pathway. The effects of temperature and pH of the mobile phase on separation, as well as the effects of the ED potential and pH of the mobile phase on detection have also been studied.

2. Experimental

2.1. Apparatus

Liquid chromatography was performed using a Model 332 gradient HPLC system (Beckman Instruments, Fullerton, CA, USA) which consisted of an Altex 110 A pump, a Model 210 injector, a Model 420 controller and an Ultrasphere-ODS reversed-phase column (250 × 4.6 mm I.D., average particle size 5 μm). A Model 153 ultraviolet detector (254 nm) was used for the determination of tryptophan. A LC-3A electrochemical detector (Bioanalytical Systems, West Lafayette, IN, USA), equipped with a glass carbon working electrode and a Ag/AgCl reference electrode, was used for the determination of 5-HTP, 5-HT, 5-HIAA, HVA and MHPG. The working electrode was set at + 0.6 V versus the reference electrode for routine analysis. The ultraviolet and electrochemical detectors were connected in series, with the electrochemical detector downstream.

2.2. Reagents

Standards were purchased from Sigma (St. Louis, MO, USA). Stock solutions (0.2–2.5 mmol/l; 0.2 mmol/l for MHPG, 2.5 mmol/l for tryptophan) were made up in distilled water with 0.1% ascorbate added. Diluted standards were made up daily in 0.1 *M* HCl. Methanol and other chemicals were obtained from commercial sources.

2.3. Mobile phase

A 0.1 *M* sodium acetate solution containing 0.1 m*M* Na_2EDTA was mixed with methanol in the ratio of 92:8 (v/v). The apparent pH was adjusted to 5.30 ± 0.02 with acetic acid, and the mobile phase was degassed under vacuum immediately before use. The flow-rate was 1.2 ml/min.

2.4. Samples

CSF samples were obtained from fourteen children with tuberculous meningitis (age ranged from 4 months to 8 years; mean ± S.D. = 3.2 ± 2.6 years), including four females and ten males. Lumbar punctures were done between 8:30–10:30 a.m. after 14–16 h of fasting. The first 2 ml of CSF were collected for determination of glucose, protein, chlorides, cell counts and bacteriological test. Subsequently 0.5 ml of CSF were collected, immediately stored at −70°C, and assayed within half a year. Prior to the study none of the examinees had ingested any compound that would affect monoamine metabolism. Parents of the children had been informed of the nature of the experiment and had voluntarily given their consent. Blood contaminated samples were discarded.

2.5. Method

CSF samples were thawed at room temperature and filtered through a 0.3-μm filter. A 50-μl aliquot of CSF was directly injected onto the chromatographic system. The compounds were

quantitated by peak-height measurement and single point standards were used.

3. Results

3.1. Evaluation of the chromatographic method

Chromatograms of 5-HTP, MHPG, 5-HIAA, HVA, 5-HT and tryptophan standards and of a CSF sample are shown in Fig. 1A and B. The retention times, detection limits, precision and recoveries for the compounds are listed in Table 1. Precision and recovery tests were performed with pooled CSF samples. Recoveries were calculated at six known concentrations of each standard (0–20 nmol/l for 5-HTP and 5-HT; 0–300 nmol/l for MHPG, 5-HIAA and HVA; 0–2.5 μmol/l for tryptophan) added to pooled CSF samples.

Good linearity was observed over the concentration range tested (from detection limit to 70 nmol/l for 5-HTP and 5-HT; to 200 nmol/l for MHPG; to 1000 nmol/l for 5-HIAA and HVA; to 10 μmol/l for tryptophan), the correlation coefficients being 0.997 for MHPG and 0.999 for the other compounds.

The effects of temperature and eluent pH on the capacity factors are shown in Figs. 2 and 3. The concentration of methanol in the mobile phase showed comparable influence on the capacity factors in the range of 8–16% (2% intervals).

The effects of the applied potential on the ED response of the compounds and on the background current are shown in Figs. 4 and 5. The influence of the pH of the mobile phase on the ED detector response is shown in Fig. 6.

3.2. 5-HTP, MHPG, 5-HIAA, HVA, 5-HT and tryptophan in CSF

A chromatogram of a human lumbar CSF sample is shown in Fig. 1B. The identities of the peaks observed in CSF were confirmed by chromatography of samples with solvent systems containing 8–16% methanol (2% intervals), and at different eluent pH values (pH 3–6, intervals of 0.5 pH units). The sample peaks always

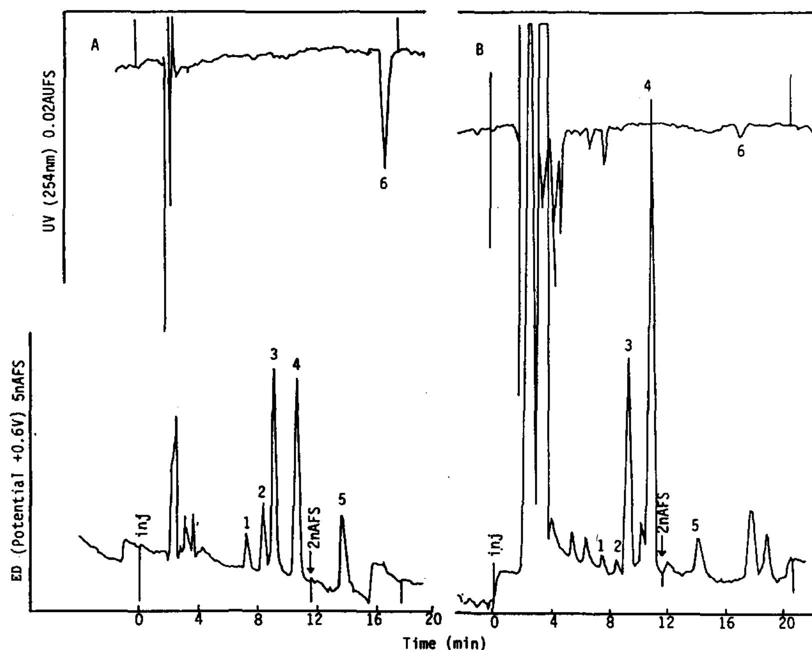

Fig. 1. Chromatograms of (A) standards and (B) a CSF specimen. Peaks: 1 = 5-HTP, 2 = MHPG, 3 = 5-HIAA, 4 = HVA, 5 = 5-HT, 6 = tryptophan. Chromatographic conditions are described in the text. Room temperature 18°C.

Table 1
Retention time, detection limit, precision and recovery for each compound

Compound	Retention time (min)	Detection limits (nmol/l)	Precision (C.V.,%)		Recovery (%)
			Within-day ($n = 8$)	Between-day ($n = 6$)	
5-HTP	7.3	1.0	2.7	5.6	97.7 ± 7.0
MHPG	8.5	2.3	5.3	6.0	109.5 ± 3.0
5-HIAA	9.2	1.3	3.1	4.6	97.9 ± 4.1
HVA	10.6	1.7	2.6	2.8	95.0 ± 4.1
5-HT	13.8	2.0	4.7	11.8	101.1 ± 11.6
Try	17.0	330	2.0	9.2	90.8 ± 9.6

Based on a 50-μl injection, signal-to-noise ratio of 2.0.

coeluted with the appropriate standards. The electrochemical detection showed maximum and half-height response at the same oxidative potentials for peaks in the CSF samples and standard solutions (0.4–0.9 V, 0.1 V intervals).

The concentrations of 5-HTP, MHPG, 5-HIAA, HVA, 5-HT, and tryptophan measured in CSF from 14 children are shown in Table 2.

4. Discussion

The presented method which can simultaneously determine tryptophan and the serotonin-related metabolites of tryptophan, as well as the main dopamine metabolite HVA and norepinephrine metabolite MHPG, with high sensitivi-

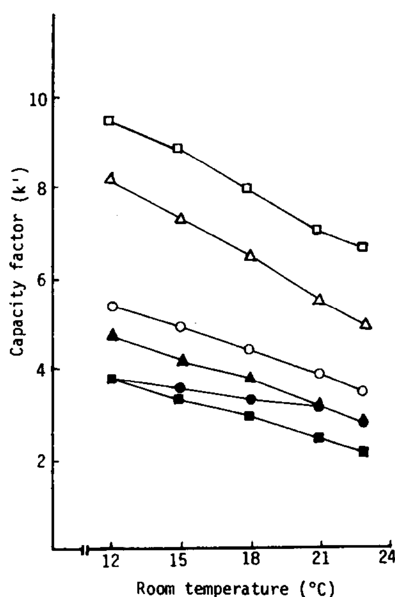

Fig. 2. Effect of room temperature on capacity factors of tryptophan(□), 5-HT(△), HVA(○), 5-HTP(■), 5-HIAA(▲) and MHPG(●). Chromatographic conditions are described in the text. Nitric acid (0.01 mol/l) was used as an unretarded compound for calculation of capacity factors.

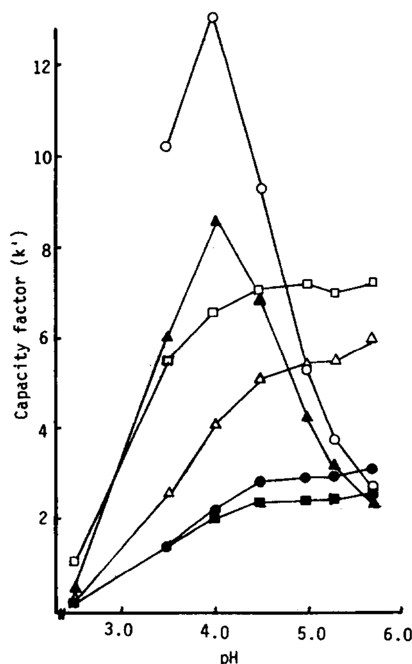

Fig. 3. Effect of eluent pH on capacity factors of the compounds. Symbols as in Fig. 2. Chromatographic conditions are described in the text except the changes in eluent pH.

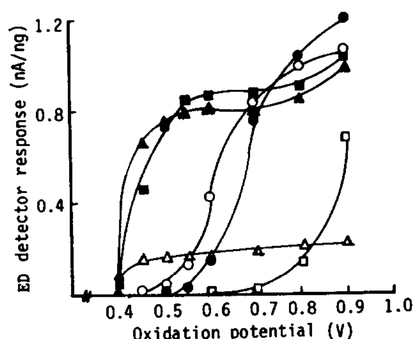

Fig. 4. Effect of applied potential on ED response of the compounds. Symbols as in Fig. 2.

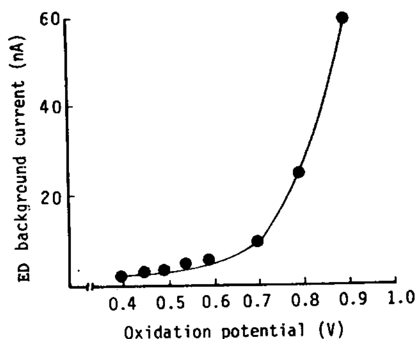

Fig. 5. Effect of applied potential on ED background current.

ty and precision, will hopefully facilitate the measurement of monoamine metabolite concentrations in CSF as indicators of monoaminergic neuronal activity in the central nervous system in clinical neuropsychiatric studies. It is a relatively simple method: no sample pretreatment is needed; it takes only ca. 20 min to run a sample and all chemicals are inexpensive.

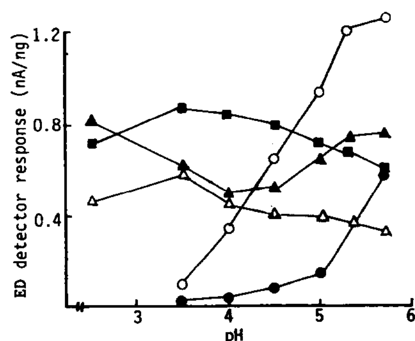

Fig. 6. Effect of eluent pH on ED response of the compounds. Symbols as Fig. 2.

Table 2
Concentrations of tryptophan, serotonin related metabolites of tryptophan, HVA and MHPG in CSF of 14 children with tuberculous meningitis

Compound	Concentration (mean ± S.D.)	Range
Trp	1.9 ± 1.1	0.4–3.9
5-HTP	7.7 ± 5.7	1.5–25.0
5-HT	4.7 ± 1.3	0–7.3
5-HIAA	179 ± 86	55–348
HVA	472 ± 245	53–830
MHPG	23.7 ± 7.6	4.9–42.7

μmol/l for tryptophan, nmol/l for others.

It is possible to influence the retention behavior of the compounds by controlling the eluent pH. The pH-dependent retention behavior of the compounds may differ depending on the buffers used in the mobile phase, as reported in the literature [7–11]. Acetate buffer is often used in the mobile phases in previously reported methods for determination of monoamine metabolites [3–5,12], with or without ion-pairing reagents and other organic acids. In the present study, using acetate buffer, the retention times of all compounds increased with increasing eluent pH below pH 4. Above pH 4, an increase in eluent pH decreased the retention times of HVA and 5-HIAA sharply, while the retention of MHPG, 5-HTP, 5-HT and tryptophan remained relatively unchanged. Satisfactory separation was obtained only with the acetate buffer system.

Variation in room temperature may change the retention times of the compounds. This may influence the separation, especially when there is no column temperature controller. As shown in Fig. 2, complete separation has been obtained at a room temperature of 18°C, with an eluent pH of 5.30. Above 18°C, a lower eluent pH was required to separate 5-HIAA and MHPG. Good resolution could be achieved with a stable room temperature at any point between 18 and 24°C.

Voltammograms have been used for the identification and selective detection of monoamine

metabolites [2,7]. Tryptophan exhibits an ED response only when the working potential is higher than 0.7 V. However, when the ED potential is higher than 0.7 V the background current is largely increased, which consequently influences the detection of 5-HT and 5-HTP. Thus the potential may be set at 0.6–0.7 V for routine analysis. At 0.7 V, peaks of HVA and 5-HIAA often run off-scale, due to their relatively high concentrations in CSF, especially in children. Thus, 0.6 V was chosen in the present method, and a UV absorbance detector was used for detection of tryptophan. Even though 0.6 V is in the middle of the steepest part of the voltammogram for HVA and MHPG, a precision test showed that it is acceptable for quantitation.

The concentrations of tryptophan, 5-HTP, 5-HT, 5-HIAA, HVA and MHPG in CSF obtained are similar to those reported in most previously published papers [3,7,13–16]. A wide range of values for 5-HT have been reported and the true levels have been questioned [12]. In the present study, we detected a 5-HT peak in all but two CSF samples. It behaved identical to the standard 5-HT when the separation and detection conditions were changed. Recently, we confirmed this in three remaining CSF samples by using a new LC-17A ED detector with a LC-4B controller, with the same sensitivity as employed by Anderson et al. [12]. The values for 5-HIAA and HVA in the present study are higher than those for adults found in the literature [3,16]. We have also found a decreasing tendency with age for the concentrations of 5-HIAA, HVA and 5-HT, even if it is not statistically significant. The two cases in which 5-HT could not be detected in CSF concern children over five years old. The effect of patient age on CSF concentrations of monoamine metabolites has been reported previously [13–15]. Thus, it is necessary to indicate the age of the subjects for evaluation of the true level of 5-HT in CSF in future studies. The status of the disease, which may influence the 5-HT level (both increase or decrease are possible), should also be considered.

Acknowledgements

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