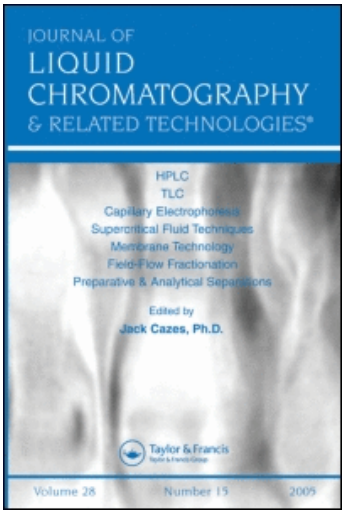

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A SIMPLE HPLC METHOD IN MONITORING CATECHOLAMINES, TRYPTOPHAN, AND THEIR METABOLITES IN CSF OF RATS: A ROLE OF DOPAMINE AND NORADRENALINE IN FOOD INTAKE

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ABSTRACT

An HPLC method in an isocratic mode with electrochemical detection is applied to monitor the levels of catecholamines, tryptophan and their metabolites in cerebrospinal fluid within 15 min. The method of is reproducible, stable and sensitive and offers an opportunity to study alterations caused by physiological changes. Deprivation of food reduced the levels of dopamine and its metabolites and increased the level of noradrenaline in the cerebrospinal fluid of rats and subsequent ingestion of food or intra-peritoneal injection of cholecystokinin octapeptide restored the levels to those of freely fed rats.

INTRODUCTION

The monoamines, dopamine (DA), noradrenaline (NA), adrenaline (A) and 5-hydroxytryptamine (5-HT) serve as chemical

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neurotransmitter in the central nervous system (CNS) [1,2]. Monoamine-containing neurons in the CNS form several anatomically distinct systems which innervate many brain regions [1]. The functional status of these systems can be studied by measurement of amines and their metabolites i.e. DA and its metabolites 3-4,dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA), NA and its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) and tryptophan and its metabolites 5-HT and 5-hydroxy-indole-3-acetic acid (5-HIAA) in the brain tissue samples or in the extracellular i.e. the cerebrospinal fluid (CSF) [3].

In view of the enormous interest of researchers and clinicians in the functional roles of the monoamines in neural pathophysiology, simple and reliable methods of quantitation are required. We have recently shown GABA, Trp and enkaphalin involvement in sexual activity and lactation by various HPLC assays [4-6] and here we describe a method based on the HPLC-electrochemical detection for monitoring alterations in the CSF in response to a physiological challenge, deprivation of food and subsequent feeding or injection of cholecystokinin octapeptide (CCK-8), a physiological satiety peptide in rats [7, 18].

MATERIAL AND METHODS

Materials

All reference compounds of high purity were obtained from Sigma Chemical Company, St. Louis, MO, USA. HPLC grade methanol was obtained from Rathburn Chemical Company, Walkerburn, Scotland. 5-sulfosalicylic acid, sodium bisulphate, EDTA (disodium salt), disodium hydrogen phosphate, sodium dihydrogen phosphate and orthophosphoric acid (85%), all reagent were of Anal-R quality

and obtained from Merck,Darmstadt, Germany. 1-octane sulphonic acid (sodium salt) "HPLC grade" was obtained from Fison Scientific equipment, Loughborough, England.

Individual 1 µM standard stock solutions were prepared in distilled deionized water containing 0.1 M sodium bisulphate as an antioxidant. These standards were further diluted to 1 nmol/L concentration. Standard of Trp and 5-HIAA were at all time covered with foil paper to protect them from sunlight. Dihydroxy benzoicacid (DHBA) was used as an internal standard.A standard mixture containing 2 µmol/l of each substance was prepared and diluted to 0.01, 0.02, 0.05 and 0.01 µmol concentrations to construct the calibration curve . All the standards were kept at -70 °C when not in use.

Chromatographic system

The system consisted of an HPLC pump from LKB, Bromma, Sweden (Model no. 2150) and a syringe loading rotatory injection valve from Rheodyne, Cotati, CA, USA (Model no 7125) with a 20 µl sample loop. Separation was conducted on a 5 µm Microsorb, C_{18} column (150 x 4.6 mm i.d.). A pre-column (4 x 4 mm i.d.) with similar material was inserted between the analytical column and injector. Both columns were obtained from Rainin instruments, Woburn, MA, USA. Electrochemical detection (EC) of monoamines was accomplishing by using an LC-4A amperometric detector with a glassy carbon electrode. (TL-5A) held at +900 mV vs. Ag/AgCl as a reference electrode, obtained from Bioanalytical Systems (BAS) West Lafayette, USA. The EC signal was recorded on a chromatograpac C-R6A data automation system obtained from Shimadzu corporation, Kyoto, Japan.

The mobile phase consisted of 12% methanol (V/V) in 0.05 M disodium hydrogen phosphate-phosphoric acid (pH 3.7). Octane

sulphonic acid and EDTA were added to a final concentration of 1.2 mM and 0.15 mM respectively. The mobile phase was filtered through 0.45 µm filter paper (Millipore, USA) and degassed under vacuum by ultrasonic filtration. Helium gas was bubbled through the mobile phase for an hour before use. All separation were performed at a flow rate of 1.2 ml/min at ambient temperature. After 3 days of use, a new mobile phase was prepared.

Animals and collection of CSF samples

Male Wister rats (Möllegård Breeding Laboratories, Ejby, Denmark) approximately 350 grams body weight were maintained individually in an air conditioned colony room in which the lights were off between 12^{00} and 24^{00} hours. The rats were deprived of food for various periods of time but water was freely available. CSF samples were obtained from the cistern magna as described earlier [7]. Six rats were included in each group.The CSF samples were centrifuged at 3000 g for 15 min at +4 ^{0}C and the clear supernates were separated and kept at -70 ^{0}C. An aliquot of 50 µl of CSF was thoroughly mixed with 25 µl each of 4% SSA and internal standard DHBA (1 µM). The mixture was centrifuged at 2500 g for 15 min at +4 ^{0}C. An aliquot of 50 µl of the supernatant was mixed with equal volume of 0.05 M phosphate buffer, (pH 3.7) containing 0.1 M sodium bisulphate. The mixture was stored at -70 ^{0}C if not analysed immediately.

RESULTS AND DISCUSSION

Chromatographic methodology

Figure 1 shows a typical chromatogram of (A) the standard mixture (B) CSF from a freely-fed rat (C) after 2 and (D) 6 hours of deprivation, demonstrating the baseline separation of most of the monoamine and tryptophan metabolites. Most of these substance are oxidized at potential < +900 mV vs. Ag/AgCl and an

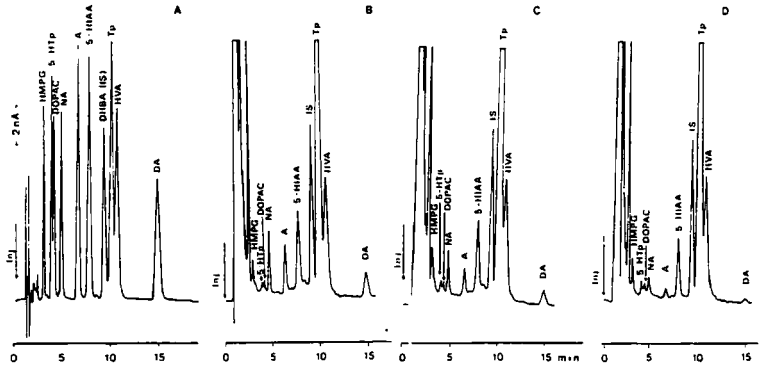

Figure 1. HPLC separation of (A) a standard mixture containing 0.2 µmol/L of each amine, (B) CSF from a freely-fed rat, and after (C) 2 and (D) 6 hours of deprivation of food.

increase in the potential increased the sensitivity of EC detector but because of baseline disturbance resulting from endogenous compounds in CSF samples and the risk of reducing the performance of the EC detector, an applied potential of +900 mV was used. This provided adequate sensitivity to accurately quantitate all substance. Identification of each substance was achieved by comparing the retention time with standard and by standard addition .Chromatographic peaks were quantitated on the basis of peak area and its ratio to the internal standard.

The linear relationship was obtained between 0.01-0.2 µM concentrations versus integrated areas for all the substances. The linearity remained valid over a wide range of concentrations of both the standards and the spiked CSF samples giving r^2 (correlation coefficient) from the regression data equal to unity for all substances.

Running 10 consecutive chromatograms on a standard mixture containing 0.1 μM of each substance and a CSF sample the coefficient of variation for retention times of each substance was < 1% whereas integrated areas showed a coefficient of variation between 0.5% and 2.5%. The within-day precision of the quantitative results gave a coefficient of variation < 1% for all substances and day-to-day precision gave a coefficient of variation < 2%. The rate of recovery of the internal standard added to a CSF sample prior to the deproteinization procedure showed the recovery at 10 pM concentration was 87%-92% and at 100 pM/L was 92%-99% for all substances.

Although in the recent years, various HPLC methods with EC or fluorescence detection based on isocratic elution for quantitation of monoamines, Trp and their metabolites have been successfully applied in varieties of biological samples [8-13]. However, most of these methods of analysis have either long time of analysis or tedious sample preparation steps including extraction with organic solvents which results in low recoveries.

Inconsistent results on the levels of monoamines, Trp and their metabolites have also been caused by sample instabilities and degradation on storage. Various studies have shown that the causes of decomposition of amines are light [14], temperature [15] and acids [16]. Failure to standardize storage times and condition within and between studies may therefore result in invalid results. In our study, we adopted a deproteinization procedure with 4% SSA under temperature-controlled conditions in the presence of sodium bisulphate to stabilize these substances. Using this procedure the recovery was better than 90%. The addition of phosphate buffer, to deproteinized samples also

TABLE 1

CSF levels of catecholamines, tryptophan and their metabolites in rats under freely-fed and food deprivation of 1 hour, 6 hours, 24 hours and 48 hours. All the values are expressed as µmol/L and as mean ± standard deviation (SD).

Subtances	Freely-fed	Deprivation of food			
		1 hour	6 hours	24 hours	48 hours
MHPG	0.039±0.015	0.041±0.011	0.036±0.01	0.042±0.01	0.040±0.01
DOPAC	0.097±0.026	0.12±0.04	0.10±0.01	0.07±0.02*	0.07±0.02*
NA	0.53±0.17	0.54±0.15	0.96±0.12**	0.98±0.12**	0.92±0.3*
A	0.12±0.07	0.09±0.03*	0.10±0.02	0.11±0.02	0.10±0.02
5-HIAA	0.63±0.23	0.67±0.18	0.66±0.21	0.68±0.31	0.69±0.21
TRP	1.83±0.36	1.93±0.42	1.87±0.2	2.01±0.3	1.96±0.3
HVA	0.11±0.04	0.10±0.03	0.08±0.02	0.08±0.01	0.008±0.01
DA	0.21±0.03	0.22±0.04	0.02±0.01***	0.02±0.01***	0.02±0.01***

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

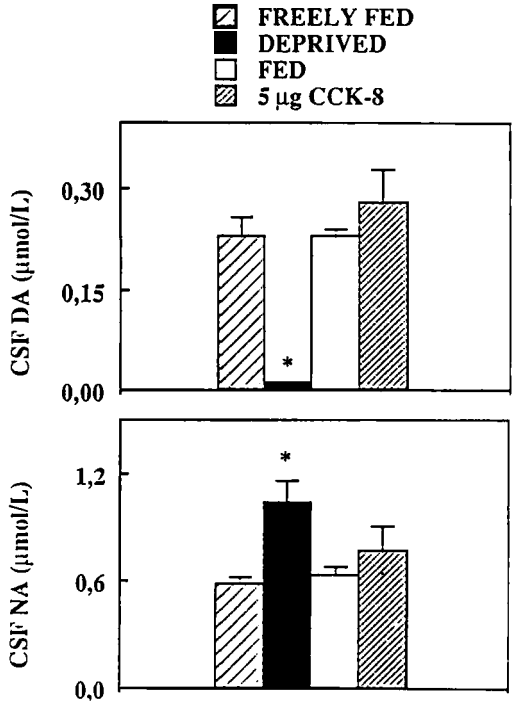

Figure 2. Levels of DA and NA in CSF of male rats. The rats were freely-fed, deprived of food for 6 hours or deprived of food for 6 hours and allowed to eat for 1 hour or injected intraperitoneal with 5 µg CCK-8. 10 minutes before the collection of CSF. Values are expressed as mean ± standard error of the mean (SEM). * $p < 0.01$ compared to freely-fed rats served as control.

retard decompositions as most of these substances are known to decompose under acidic conditions [17].

Physiological Validation

Table 1 shows the effect of different periods of deprivation of food on the levels of the monoamines and their metabolites in the CSF. These did not change after one hour of deprivation.

However a six hour period of deprivation caused a reduction in the concentration of DA and an increase in the concentration of NA. Longer periods of deprivation also decreased the concentration of the DA metabolites whereas no alterations occurred in the concentration of MHPG,.Trp and 5-HIAA levels remained unaltered even with a long period of deprivation of food.

Figure 2 shows that the reduced level of DA and the increased level of NA, which was caused by six hours of deprivation of food was reversed either by one hour of feeding by intraperitoneal injection of 5 µg CCK-8. This dose of CCK-8 decreased the intake of pellets (3.2 ± 0.5 g) compared with NaCl-injected control (5.2± 0.4 g $p < 0.01$) during a 1 hour test period.

These experiments show that a mild physiological challenge i.e. deprivation of food after six hours, causes alterations in the concentration of DA and NA in the CSF and that these are reversed by one hour of feeding or intraperitoneal injection of 5 µg CCK-8. This shows the usefulness of the present methodology for physiological studies and is in line with the suggested role of DA [7] and NA [19, 20] in the mechanisms for action of CCK-8 in the control of food intake. However, pharmacological experiments are needed to investigate these mechanisms in further details. The present method of analysis is reproducible, stable and sensitive and requires 15 min to quantitate catecholamines and their metabolites along with Trp and 5-HIAA in the same run.

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