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DETERMINATION OF NEUROTRANSMITTER SYSTEMS IN HUMAN CEREBROSPINAL FLUID AND RAT NERVOUS TISSUE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ON-LINE DATA EVALUATION

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SUMMARY

We describe an improved high-performance liquid chromatographic method for the determination of tyrosine, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, tryptophan and homovanillic acid in cerebrospinal fluid and nerve tissue, using the new microbore cartridges with 5 μm average particle size. The first four substances are quantified fluorometrically and the last two electrochemically. Both detectors are connected to the same integrator through a relay which can be switched as required. Data are collected in an on-line personal computer and evaluated statistically. An improvement in the method for extraction and separation of catecholamines is also reported.

INTRODUCTION

In the course of studies on central nervous system function we needed a high-performance liquid chromatographic (HPLC) method for detecting changes in the catecholamine and indoleamine systems, i.e. one able to measure not only the neurotransmitters themselves but also their precursors and metabolites. Finding a satisfactory single system presented a difficult problem, for two reasons. Firstly, the polarities of the compounds differ widely. Secondly, baseline separation and sharp peaks are required for maximum accuracy, so that elution times should not be too short or too long. Most of the systems described in the literature are either not suitable for all the substances which interested us or do not fulfill this second requirement (e.g. refs. 1—7). Gradient elution with increasing proportions of organic solvent should be the ideal system; however, in contrast to Krstulovic et al. [8], we found this to be

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incompatible with amperometric electrochemical detection and even to a lesser extent with fluorometric detection. We therefore decided to use two systems. Catecholamines were determined with a modification of the usual alumina extraction method (e.g. refs. 9—11). For the other compounds (5-hydroxytryptamine, 5-hydroxyindoleacetic acid, tryptophan, tyrosine, homovanillic acid) we developed a direct injection method with the following innovations:

(1) Microbore cartridges were used (internal diameter 2.1 mm, 5 μm average particle size), which operate with a low flow-rate, requiring greatly reduced amounts of mobile phase. The advantage of the cartridge system is that the column length can be tailored without difficulty to suit the required separation.

(2) The first cut from the column was diverted into the waste before the electrochemical detector, thus preventing an overload and disturbance of the baseline when extracts were injected.

(3) An electrochemical detector and a fluorometric detector were connected to the same low-price one-channel integrator through a relay, so that all the peaks, including the amino acids tryptophan and tyrosine, could be quantified as accurately as possible.

(4) The chromatographic data were stored on discs on-line with the aid of an Apple IIe computer. Software was developed for transferring data to statistical programs either in the personal computer, or in the Univac 1100 computer for more advanced evaluation.

MATERIALS

Chemicals were obtained from the following sources: 5-hydroxytryptamine creatinine sulphate (5-HT) from Serva (Heidelberg, F.R.G.); 5-hydroxyindoleacetic acid (5-HIAA), L-tyrosine (Tyr), *n*-octyl sodium sulphate, ethylenediaminetetraacetic acid disodium salt (EDTA, Titriplex III) and aluminium oxide 90 (active neutral for column chromatography) from E. Merck (Darmstadt, F.R.G.). L-Tryptophan (Trp), homovanillic acid (HVA), dopamine hydrochloride (DA), L-adrenaline-D-hydrogentartrate (A), and L-noradrenaline hydrochloride (NA) from Fluka (Buchs, Switzerland); 3,4-dihydroxybenzylamine hydrobromide (DHBA) from Aldrich Europe (Beerse, Belgium). Acetonitrile and methanol (both chromatographic grade, Merck) were used as supplied. All solutions were prepared in water that had been double-distilled in glass.

Chromatographic system A (for Tyr, 5-HT, 5-HIAA, Trp and HVA)

A solvent delivery pump (LC 414, Kontron Analytic, Zurich, Switzerland) was used in conjunction with an automatic sampler (MSI 660, Kontron) equipped with a 10-μl injection loop. Pulseless solvent delivery was achieved with a membrane-type damper (Portmann, Therwil, Switzerland). The column system, which was at room temperature, consisted of three commercially available reversed-phase cartridges in series, all with internal diameter 2.1 mm. The total length was 350 mm (RP-18 Spheri 5, 5 μm spherical particle size, 30 mm + 100 mm + 220 mm; Brownlee Labs., Santa Clara, CA, U.S.A.). Two detectors were used; upstream a fluorescence detector (LC fluorometer FS 970,

Schoeffel Instruments, Kratos, Trappenkamp, F.R.G.) with a wavelength of 225 nm for excitation and a band pass filter 7-54 for emission; downstream an electrochemical detector (656 VA, Metrohm, Herisau, Switzerland) with a glassy carbon working electrode set at a potential of 0.75 V versus the Ag/AgCl reference electrode. The output of the fluorometer was changed from 10 mV to 1 V using an FSA 987 computer output, and both detectors were connected via a relay to the same integrator (HP 3390 A, Hewlett-Packard, Avondale, PA, U.S.A.). The relay was controlled by the integrator through an intermediate 19400 A sampler/event control module (Hewlett-Packard). By programming the integrator the relay could be switched at any desired time so that either the output of the electrochemical detector or that of the fluorometer could be recorded by one integrator. Between the two detectors an automatic Rheodyne 7040 valve was inserted, which allowed the automatic cut-off of the effluent after the fluorescence detector. This was also time-controlled by the integrator. Mobile phase A contained sodium dihydrogen phosphate (0.1 *M*), EDTA (0.08 m*M*) and *n*-octyl sodium sulphate (0.025 m*M*) brought to pH 3.5 with phosphoric acid. It was filtered through a 0.4-μm filter and acetonitrile was added to a final concentration of 8%. It was degassed by stirring under vacuum (water pump) for 10 min. The flow-rate was 0.3 ml/min, at a pressure of about 175 bars.

Chromatographic system B (for catecholamines)

This was essentially as described above, the Kontron sampler being replaced by a Wisp 710 B (Waters Assoc., Milford, MA, U.S.A.). The electrochemical detector was set at 0.65 V and the fluorometer and valve were omitted. The stationary phase was an RP-18 Spheri 5 column, 100 mm × 4.6 mm (Brownlee Labs.), average particle size 5 μm, and the mobile phase was disodium hydrogen phosphate 6.7 m*M*, citric acid 13.3 m*M*, *n*-octyl sodium sulphate 2.5 m*M*, EDTA 0.05 m*M*, methanol 13—15% depending on column condition, pH 3.3 [10]. The flow-rate was 1.3 ml/min. The mobile phase was recycled. This had two advantages: firstly, the stability of the electrochemical detector was improved; and secondly, the amount of mobile phase used was greatly reduced (to 2 l in three weeks; even less than would be required with a microbore system).

Preparation of extracts

Human cerebrospinal fluid (CSF) samples were obtained from the Neurological Clinic, Kantonsspital, Basel, and cooled in ice until they reached the laboratory. They were treated with 1 *M* perchloric acid (10:1, v/v) to precipitate protein, then centrifuged for 25 min at 4°C and 9000 *g* in a Sorvall RC-5 Superspeed refrigerated centrifuge (Dupont, Newton, CT, U.S.A.). The supernatant was either injected directly (system A, 10 μl) or stored at —80°C for not longer than one week. Samples stored before perchloric acid treatment could be kept for up to three months. In both cases the thawed samples were recentrifuged for 25 min as above before injection (10 μl).

Lewis rat brain or spinal cord regions (30 mg to 1.3 g) were isolated and stored at —80°C until used. They were homogenized with cooling in ice in 0.1 *M* perchloric acid (1:10, w/v) with an ultrasonic cell disrupter, either a micro-

model from Kontes (Vineland, NJ, U.S.A.) or a Sonicator W 375 (Heat Systems—Ultrasonics, as supplied by Kontron) depending on the amount. For catecholamine determinations DHBA (final concentration 10^{-6} *M*) was added as internal standard and 0.01% sodium bisulphite as antioxidant. The homogenates were centrifuged as described above. A portion of the supernatant was removed for catecholamine isolation and determination in system B, and the rest stored at −80°C for not longer than a month. After thawing it was recentrifuged as above before direct injection (10 μl) into system A.

Analysis and quantification of Trp, 5-HT, 5-HIAA, HVA, Tyr

Direct injection of CSF and homogenates into system A caused no problem with the fluorometer. However, when the electrochemical detector was set to a sensitivity sufficient for our measurements (usually 5 nA/V) there was an overload as the initial peak reached the detector, due to water-soluble, easily oxidizable cell constituents, such as ascorbic acid. To prevent this, the first part of the efflux was diverted as described above before reaching the electrochemical detector, which greatly improved the baseline characteristics. In the concentration ranges present in our extracts, 5-HT can be determined either electrochemically or fluorometrically. 5-HIAA and HVA must be determined electrochemically if any degree of accuracy is required, whereas with 0.75 V potential Trp and Tyr must be determined fluorometrically. (They can also be determined electrochemically when the detector potential is higher than 0.9 V; in our experience, however, the system is then more unstable and much less specific.)

Retention times are in the order Tyr < 5-HT < Trp < 5-HIAA < HVA. Therefore, before injection, the relay was set so that the integrator was connected to the fluorometer until the three peaks Tyr, 5-HT and Trp had been recorded (about 14 min). The relay was then switched so that the integrator recorded the output from the electrochemical detector for the rest of the chromatogram (peaks 5-HIAA and HVA). Because the substances are present in the extracts in greatly varying concentrations, the integration attenuation was sometimes changed according to the peak size, which improved the reproducibility of the determination. Quantitative determinations were made by comparing the peak areas of the samples with those of standard solutions using the external standard calibration program of the integrator. Stock solutions in water (10^{-3} *M*) were made fresh every week, stored at 4°C and diluted as required. Because no cooled sampler was available, all solutions and extracts were brought to room temperature only a short time before injection. The detectors were allowed to stabilize for 1 h each morning before use, and were re-calibrated if necessary with two or three standard solutions. The relationship between peak area and amount injected was found to be linear for each substance in the concentration range in which it is present in nervous tissue and CSF, except for Tyr which showed a plateau at higher concentrations.

Extraction and quantification of catecholamines

A suspension of aluminium oxide (15 mg/ml) in Tris—HCl buffer [tris(hydroxymethyl)aminomethane 0.6 *M*, EDTA 0.05 *M*, pH 8.6] was stirred

magnetically for 15 min. Portions (1 ml) were pipetted during stirring into plastic tubes, to which the supernatants (200 µl) prepared as above were added (according to a private communication of A. Enz, Sandoz, Basel). The mixture was shaken for 15 min and centrifuged. The aluminium oxide was washed with water (3 × 1 ml) and as much as possible of the final washing water was removed. The catecholamines were eluted by agitation for 5 min in 200 µl of mobile phase B, brought to pH 2 with phosphoric acid. After centrifugation the supernatant, usually 20 µl, was injected directly into system B.

Standard solutions containing DHBA (10^{-6} *M*) and the other catecholamines ($2.5 \cdot 10^{-7}$ *M*, $5 \cdot 10^{-7}$ *M* or $7.5 \cdot 10^{-7}$ *M*) in 0.1 *M* perchloric acid were made and stored in portions at −80°C for up to six months. Every time tissue samples were extracted as described above, these three solutions (180 µl each) were taken through the same procedure. The aluminium oxide was eluted with mobile phase (200 µl) and 20 µl of the eluate were injected. The internal standard program of the integrator calculated the ratios of the peak areas of the catecholamines to those of DHBA and by comparison with these calculated the catecholamine content of the extracts.

Catecholamines are more stable than indoles at room temperature and the automatic sampler could be loaded and run overnight.

Computer evaluation

Hardware. This comprised the following components: Apple IIe computer with screen and optional printer; two floppy disc drives; 7710-A serial interface (from CCS) for the Apple; cable to connect the two RS 232-C in Apple and HP integrator.

Software. Our "Multi"-program was written in Applesoft Basic, apart from a subroutine to transfer the data coming from the integrator to a temporary store in the Apple's memory. This had to be written in Assembler (the machine language) because Basic is not fast enough to handle all the in-coming data. Where possible, Basic is preferable as it is easier than Assembler to write, read and modify. Using the Multi-program, the HPLC data can be manipulated in the following ways:

(1) Chromatograms can be stored in files (Chro-files) on floppy discs. Using two floppy discs, up to 260 chromatograms with seven calibrated peaks are available on-line. Each chromatogram is first stored temporarily in the Apple as described above, then sent to the floppy disc during the next chromatographic run.

(2) Any Chro-file or series of Chro-files can be called up on the screen or printed out.

(3) Data needed for statistical calculations can be sorted out from a series of Chro-files and sent to a single file, together with extra information if required. This new data file has a previously defined and programmed structure; e.g. an array (*A* matrix with *N* Chro-files and *M* defined variables). These data files can be evaluated using private statistics programs or commercial software packages. One of our Multi-program options creates data files which can be read by Micro-SPSS 4.1 [12]. These matrix data files can be enlarged vertically, i.e. the data from other chromatograms can be added at a later date without difficulty. Faulty or missing data which could be added later are replaced with

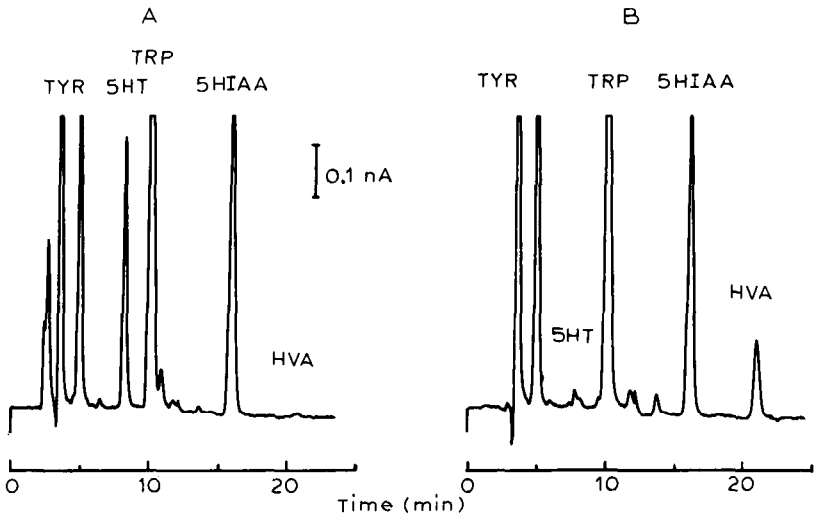

Fig. 1. Chromatograms of perchloric acid extracts prepared as described in text. Injection volume 10 μl; chromatographic conditions, system A. (A) Lewis rat spinal cord, (B) human cerebrospinal fluid.

TABLE I

RETENTION TIMES OF SOME CATECHOLAMINES, INDOLEAMINES AND THEIR METABOLITES AND PRECURSORS

Retention times are given in min.

*Compound**	*System A*	*System B*
Tyr	3.47	
NA	3.50	6.24**
DOMA	3.51	4.44**
DOPA	3.54	4.03**
A	3.65	8.90**
DOPEG	3.71	1.19**
DHBA	3.84	12.50**
VMA	4.05	1.36
DA	4.47	19.69**
5-HTP	4.88	11.51
NM	5.17	12.19
MHPG	5.25	1.83
5-HT	8.45	ca. 50
DOPAC	9.12	3.30**
Trp	10.19	
5-HIAA	16.59	5.11
HVA	20.96	6.90

*DOMA = 3,4-dihydroxymandelic acid, DOPA = 3,4-dihydroxyphenylalanine, DOPEG = 3,4-dihydroxyphenylethylene glycol, VMA = vanillylmandelic acid, 5-HTP = 5-hydroxytryptophan, NM = 3-O-methylnoradrenaline, MHPG = 3-methoxy-4-hydroxyphenylethylene glycol, DOPAC = 3,4-dihydroxyphenylacetic acid.

**Can be extracted from homogenates by Al_2O_3.

a selected "missing value". Another option of the Multi-program enables data to be arranged in a format that can be read by BMDP statistical software (on a Univac 1100) for sophisticated statistical evaluation of large amounts of data. With a special program and a corresponding hardware connection developed by Dr. Christen (URZ, Basel), data from the Apple can be sent to the Univac.

RESULTS AND DISCUSSION

System A

Fig. 1A and B shows typical chromatograms obtained by direct injection of rat spinal cord extract and human CSF into system A. The first part of the chromatogram shows the peaks recorded by the fluorometer, the second part those recorded electrochemically. As can be seen, the switching of the relay took place without greatly affecting the baseline. In developing our method we considered baseline separation to be of primary importance. As can be seen from the figures, we have obtained good separations of Tyr, 5-HT, Trp, 5-HIAA and HVA, even with extracts. The identification of peaks with the direct injection method always causes problems. We approached this in the same way as other authors [4, 12, 13]: multiple detectors (electrochemical, fluorometric), variable detector potential (0.65—0.95 V), extraction with alumina (specific for catechol derivatives) and injection into a different chromatographic system (without octyl sulphate, results not shown). The five peaks quantified can be considered to be unambiguously identified. Table I lists typical retention times of the catecholamines, indoleamines and some of their common metabolites in the two systems. In system A, catecholamines and their metabolites were eluted in the first 5.5 min, well before 5-HT. An exception was 3,4-dihydroxyphenylacetic acid (DOPAC), which eluted between 5-HT and Trp; however, as it has practically no fluorescence under our conditions, it caused no interference. The electrochemical method would be more sensitive for 5-HT [4] but as it is present in relatively large quantities in nerve extracts this is not of great importance. We have tried to obtain improved separations of CSF using other chromatographic systems, including that of Gattaz et al. [14], and columns with smaller particle size. However, our latest results with the column RP-18 Spheri 3 μm, length 100 mm (Brownlee) show that (a) the pressure is so high (160 bars at a flow-rate of 0.3 ml/min) that the column could not be much lengthened, and (b) the separation on this column is not good enough to replace our system A. As already reported by others [15], we found that Tyr, Trp, 5-HT, HVA and 5-HIAA were not stable for more than a few hours at room temperature, even in the presence of anti-oxidants, in standard solutions and to a lesser extent also in extracts. All samples were therefore brought to room temperature only a short time before injection to prevent errors. Recoveries, determined by the standard addition method, were as follows: Tyr 83%, Trp 100%, 5-HIAA 100% and HVA 100% from CSF; and Tyr 98%, 5-HT 99%, Trp 100%, 5-HIAA 100% and HVA 98% from Lewis rat spinal cord.

System B

Fig. 2 shows a chromatogram of an alumina extract from rat spinal cord

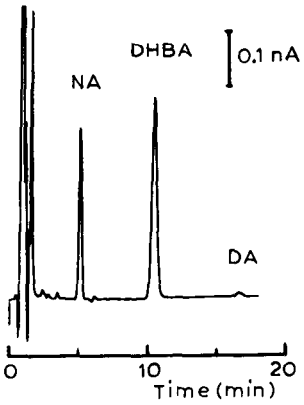

Fig. 2. Chromatogram of catecholamines extracted by alumina from Lewis rat spinal cord as described in text. Injection volume 20 μl; chromatographic conditions, system B.

TABLE II

CONTENTS OF SUBSTANCES IN RAT BRAIN AND HUMAN CSF

Results are measured in ng/g fresh weight (rat spinal cord) or ng/ml (human CSF) and expressed as mean ± S.E.M.

	Tyr	5-HT	Trp	5-HIAA	HVA	NA	DA
Rat spinal cord (n = 10)	12,978 ± 712	823.2 ± 14.9	3830 ± 129	406.6 ± 6.9	0.66 ± 0.66	559.9 ± 13.6	33.3 ± 2.02
Human CSF (n = 8)	n.d.*		372 ± 18	23.3 ± 2.2	31.8 ± 2.6	—	—

*n.d. = not determined.

homogenate injected into system B. The catecholamines NA, A, DHBA (internal standard) and DA were separated in 20 min. The separation required only a 100-mm column (5 μm) compared with 250 mm using a Bondapak RP-18 with 10 μm average particle size. Alumina extraction is specific for catecholamines, so peaks could be readily identified. Recovery rates from standard solutions were 84.7 ± 0.4% for NA, 88.9 ± 1.3% for A, 84.4 ± 2.1% for DHBA and 84.9 ± 2.2% for DA (n = 4). These high rates were only obtained when the alumina was stirred in Tris buffer prior to extraction and may be the result of better contact between the buffer and the alumina particles. By eluting the catecholamines from the alumina with mobile phase, instead of perchloric acid, the initial peak was reduced and the baseline improved without affecting the yield.

Table II shows the contents of the substances which we found in rat spinal cord and human CSF. For CSF they are comparable with those found in the literature [14, 16—18], the main difficulty here being the enormous variation found in normal persons [17]. For rat spinal cord the values for 5-HT, NA and DA are similar to those obtained by Zivin et al. [19]. In a more recent publication [20] values are expressed as pg/mg protein and are therefore not directly comparable.

Our present method, like other chromatographic methods, is a compromise. For instance 3-methoxy-4-hydroxyphenylethylene glycol (MHPG), a substance of great interest in CSF, can only be quantified with two-channel integration. Other authors [21—24] have used direct injection combined with electrochemical detection for both catecholaminergic and indoleaminergic substances. However, we chose the alumina extraction method for catecholamines because, when extracts are directly injected, large amounts of interfering non-catecholaminergic substances co-elute with the catecholamines. We found that this made baseline separation and reproducible results very difficult to obtain. In a recent publication Tjaden and de Jong [25] suggest that dual coulometric electrochemical detection may solve the problem of gradient-induced baseline shift. This could lead to an improved separation of substances in one run, which would offer an alternative to isocratic methods such as ours.

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