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COMBINED ION-PAIR EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF THE BIOGENIC AMINES AND THEIR MAJOR METABOLITES IN SINGLE BRAIN TISSUE SAMPLES

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SUMMARY

A combined procedure based on reversed-phase liquid chromatography with electrochemical detection has been developed for the determination in the picomole range of the monoamines dopamine, norepinephrine, epinephrine and serotonin, and their major metabolites 3,4-dihydroxyphenylacetic acid, homovanillic acid, 3-methoxy-4-hydroxyphenylethylene glycol, 5-hydroxyindoleacetic acid, normetanephrine, metanephrine and 3-methoxytyramine. Sample pretreatment consists of the extraction of the neutral and acidic metabolites with ethyl acetate, followed by the extraction into heptane of the catecholamines with tetraoctylammonium bromide as counter-ion in the presence of diphenylborate. The residual supernatant is directly injected in the chromatographic system for quantification of serotonin, normetanephrine, metanephrine and 3-methoxytyramine.

INTRODUCTION

A better understanding of the monoamine neurotransmission systems in the central nervous system of different species has been greatly assisted by quantitative locoregional determination of the biogenic amines dopamine (DA), norepinephrine (NE), epinephrine (E) and serotonin (5-HT), and their metabolites in single brain tissue samples. Most of the publications describing extraction procedures and regional distribution studies permit the determination of only some of these neurotransmitters and some of their metabolites from the same tissue sample [1,2]. The catecholamines NE, E and DA are usually isolated by

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adsorption onto alumina [3,4]. The neutral and acidic metabolites 3-methoxy-4-hydroxyphenylethylene glycol (MHPG), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) are most frequently extracted with ethyl acetate [5]. Normetanephrine (NMN), metanephrine (MN), 3-methoxytyramine (3-MT) and 5-HT are generally isolated by ion-exchange chromatography [6,7], yielding low extraction recoveries, possibly due to the presence of lipids or proteins in the supernatant and also to emulsification during the extraction procedure [8,9]. Some investigators have described column extraction techniques based on adsorption on Amberlite CG-50 [10,11] or Sephadex G-10 [12], all of which have drawbacks concerned with the limited number of compounds that can be extracted and with imprecise or low recovery due to difficulties of column preparation.

A new trend in the investigation of monoamine systems is the quantitative determination by direct injection of the tissue homogenate into a high-performance liquid chromatographic (HPLC) system. The advantages of this technique are its rapidity, the minimal sample preparation and the low degradation of the compounds analysed [13–15]. A great disadvantage, however, is the incomplete chromatographic separation of all the compounds, resulting in less reliable quantitative interpretations.

We describe here a combined extraction procedure permitting a selective isolation of the neutral and acidic metabolites MHPG, DOPAC, 5-HIAA and HVA with ethyl acetate, followed by a quantitative isolation of the catecholamines DA, NE and E by ion-pair extraction with tetraoctylammonium bromide (TOABr) in the presence of diphenylborate [16]. This technique is an extension of the extraction procedure proposed by Smedes et al. [17] for the determination of the catecholamines in plasma and urine. After these two consecutive extractions, the residual supernatant is directly injected in the chromatographic system, permitting an easy determination of NMN, MN, 3-MT and 5-HT. This combined procedure yields accurate and precise results and short chromatographic runs. Moreover, it allows the fast and simple determination of 5-HT and the metanephrines by injection of the tissue homogenate after the extraction of interfering compounds.

EXPERIMENTAL

Materials

The HPLC system consisted of a Varian 8500 (Varian Instruments, Sunnyvale, CA, U.S.A.) pulse-free pump equipped with a 100-μl Valco injection loop (Valco Instruments, Houston, TX, U.S.A.). The amperometric detector was a Bruker E230 equipped with an LCC 231 thin-layer electrochemical cell fitted with a glassy-carbon working electrode and a calomel reference electrode. Integration of the chromatographic peaks was achieved with a VISTA CDS 401 integrator (Varian). Homogenization of brain tissue was performed in a Potter homogenizator (type B, Braun) and samples were centrifuged in a Beckman centrifuge (type J2-21). Chromatography was performed on a Partisil-5 ODS-3 column (particle size 5 μm; 10 cm $\times$ 0.4 cm I.D.) from Whatman (Maidstone, U.K.). The

guard column (3 cm × 0.4 cm I.D.) was packed with LiChrosorb RP-18 (particle size 10 μm) from Merck (Darmstadt, F.R.G.).

Chemicals and reagents

Dopamine hydrochloride was purchased from UCB (Belgium). Norepinephrine, epinephrine, homovanillic acid and tetraoctylammonium bromide were supplied by Fluka (Buchs, Switzerland). 3-Methoxy-4-hydroxyphenylethylene glycol piperazine, 3-methoxy-4-hydroxybenzylamine (MHBA) hydrochloride, 3-methoxytyramine hydrochloride, serotonin hydrochloride hemihydrate, 5-hydroxyindoleacetic acid, 3,4-dihydroxybenzylamine (DHBA) hydrobromide, 5-hydroxy-N-methyltryptamine (5-H-NMetT) oxalate, diphenylborate–ethanolamine, di-*n*-butylamine and 1-octanesulphonic acid sodium salt were purchased from Janssen Chimica/Aldrich-Europe (Beerse, Belgium). 3,4-Dihydroxyphenylacetic acid, *d,l*-normetanephrine hydrochloride and *d,l*-metanephrine hydrochloride were supplied by Sigma (St. Louis, MO, U.S.A.) and iso-homovanillic acid (iso-HVA) was generously supplied by Roche (Basel, Switzerland). All other chemicals used were purchased from Merck (Darmstadt, F.R.G.). Deionized water was used throughout. Stock solutions at 100 mg/l were prepared by dilution of the standards in 0.01 *M* hydrochloric acid containing 0.1% disodium bisulphite (w/v) and 0.01% Na_2EDTA (w/v) and kept at 4 °C.

Chromatographic conditions

The mobile phase consisted of an acetate–citrate buffer containing 0.1 *M* sodium acetate, 20 m*M* citric acid 1-hydrate, 1 m*M* 1-octanesulphonic acid, 0.1 m*M* Na_2EDTA and 1 m*M* dibutylamine. The pH of the mobile phase was adjusted to 3.0 with concentrated phosphoric acid. The flow-rate was set a 1 ml/min, and the detector potential was +0.75 V versus the calomel reference electrode, range 20–50 nA.

The use of internal standards

Iso-HVA was added as the internal standard for the ethyl acetate extraction, DHBA was used in the catecholamine ion-pairing isolation with TOABr. MHBA and 5-H-NMetT were used as internal standards for the quantitative determination of the metanephrines and serotonin, respectively.

Assay standards

Standards were prepared by spiking 100 mg homogenate of cerebellum with each compound to give final concentrations of 1–50 ng/ml. The concentration of the internal standards was 50 ng/ml in each standard solution. The standards were taken through the entire extraction procedure.

Quantitation

Linear calibration graphs of the peak-area ratio of the standards to the internal standard, versus the concentration of the standard were constructed (range 1–50 ng/ml).

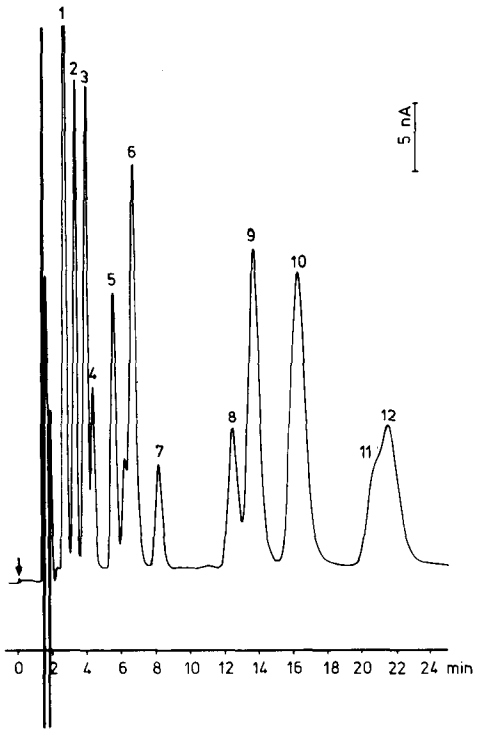


Fig. 1. Chromatogram of 100 µl of a mixture containing 100 ng/ml of each reference and each internal standard. Superposition of different substances of interest makes an exact quantitation impossible. Peaks: 1 = NE; 2 = E and MHPG; 3 = DHBA and NMN; 4 = MN; 5 = DA; 6 = DOPAC; 7 = MHBA; 8 = 3-MT; 9 = 5-HIAA; 10 = HVA and 5-HT; 11 = iso-HVA; 12 = 5-H-NMetT. Mobile phase, 0.1 *M* sodium acetate, 20 m*M* citric acid buffer containing 1 m*M* 1-octanesulphonic acid, 0.1 m*M* Na_2EDTA and 1 m*M* dibutylamine at pH 3.0, methanol 5%; flow-rate, 1 ml/min; electrochemical detection at +0.75 V, range 50 nA.

OPTIMIZATION OF THE EXTRACTION PROCEDURE AND RECOVERY STUDY

Combination of the ethyl acetate extraction followed by ion-pair extraction with TOABr

Standard mixtures at 100 mg/l of MHPG, DOPAC, 5-HIAA, HVA, iso-HVA, NE, E, DHBA, DA, NMN, MN, MHBA, 3-MT, 5-HT and 5-H-NMetT were prepared in 0.01 *M* hydrochloric acid containing 0.1% disodium bisulphite and 0.01% Na_2EDTA. Further dilution at 10, 20, 40, 100 and 200 ng/ml of mixtures containing all the compounds under study were prepared in 0.1 *M* hydrochloric acid. A pool of 1000 mg of human cerebellar cortex was homogenized in 5 ml of 1 *M* hydrochloric acid containing 0.1% disodium bisulphite and 0.01% Na_2EDTA and 5 ml of deionized water were added. Then 1.5 ml of the prepared diluted standard mixtures and an equal amount of homogenized cerebellum suspension were vigorously shaken by hand. A 1.5-ml volume of the same cerebellum suspension was used as a blank. Each sample was centrifugated at 23 000 *g* for 15 min. Even after modification of the experimental conditions, no complete separation

of all the compounds could be achieved in a single chromatographic run (Fig. 1). An interesting alternative to the direct injection of the complete homogenate appeared to be the injection of a supernatant after previous extraction of the alcoholic and acid metabolites as well as the catecholamines. The complete extraction procedure is summarized in Fig. 2.

Extraction of MHPG, DOPAC, 5-HIAA, HVA and iso-HVA with ethyl acetate (part A of Fig. 2). Table I shows that the recoveries for all the acidic and alcoholic compounds studied varied between 46.4% and 97.9% at pH 4.0. A recovery study performed previously [16] showed a very high influence of the pH on the recovery yield. The optimal pH value that permits an acceptable recovery for all compounds together was found to be pH 4.0. At this pH the recoveries for MHPG and 5-HIAA were rather low, but still reasonable precise. In order to correct for the differences in percentage recovery between the compounds and the internal standard (iso-HVA), calibration graphs were constructed by extracting the standards in the same way as the samples.

Extraction procedure of the catecholamines NE, E, DHBA and DA. The extraction procedure is summarized in Fig. 2, parts B and C. The recoveries for the compounds extracted are given in Table II.

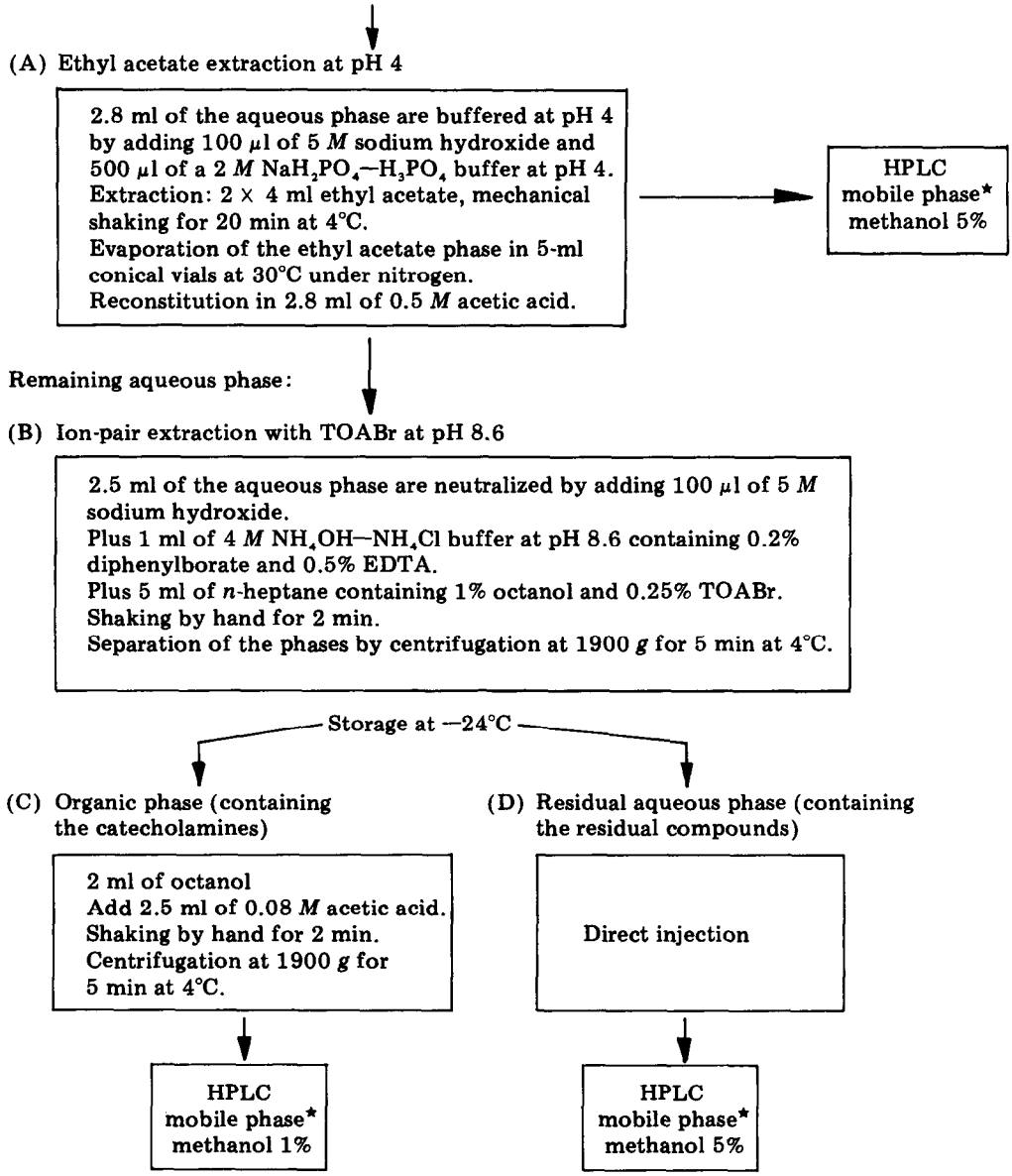
Quantitation of NMN, MN, MHBA, 3-MT, 5-HT and 5-H-NMetT by direct injection of the residual supernatant (Fig. 2, part D)

A satisfactory detection was obtained by increasing the sensitivity of the electrochemical detector from 50 to 20 nA. This led to a practical determination limit of 1.5 pmol on column for all the compounds under study. Table III gives the recoveries of all the compounds detected, after correction for the dilution factor due to the previous combined extraction.

RESULTS AND DISCUSSION

The results obtained show a satisfactory recovery for the extraction of the neutral and acidic compounds with ethyl acetate. The catecholamines are completely isolated by the ion-pair extraction procedure with TOABr in the presence of diphenylborate. These recoveries are far superior to those obtained by adsorption on alumina, ranging between 50% and 70% according to the method developed by Anton and Sayre [18,19]. Direct injection of the residual supernatant yields very high recoveries for NMN, MN, MHBA, 3-MT, 5-HT and 5-H-NMetT, all compounds that were not extracted by the previous combined extraction procedure. This proves that the whole procedure causes minimal degradation of the compounds analysed. Residual amounts of MHPG and 5-HIAA, two compounds that are only partially extracted with ethyl acetate at pH 4.0, are also recovered in the residual supernatant after direct injection onto the HPLC system but without interference with an other substance of interest. Fig. 3 shows separately the chromatograms of the ethyl acetate extraction, the ion-pair extraction and the residual supernatant. The graphs are remarkably simple. After direct injection of the residual supernatant the chromatograms shows a complete separation of all the remaining compounds with the mobile phase described previously. The dis-

Homogenization of the tissue sample in 1.5 ml of 1 *M* hydrochloric acid containing 0.1% $Na_2S_2O_5$ and 0.01% Na_2EDTA and 1.5 ml of deionized water.
Centrifugation of the homogenized brain samples at 23 000 *g* for 15 min at 4°C.
Supernatant is washed with 5 ml of *n*-hexane by mechanical shaking for 20 min at 4°C, followed by centrifugation (1900 *g* for 5 min at 4°C).



*Mobile phase = acetate—citrate buffer containing 1 m*M* 1-octanesulphonic acid at pH 3.0; methanol concentration is specified for each extraction.

Fig. 2. Schematic survey of the combined extraction procedure for brain tissue samples.

TABLE I

ETHYL ACETATE EXTRACTION OF MHPG, DOPAC, 5-HIAA, HVA AND iso-HVA AT pH 4.0

Compound	Recovery (mean ± S.D., $n = 10$) (%)
MHPG	46.4 ± 5.6
DOPAC	70.9 ± 4.1
5-HIAA	47.1 ± 5.8
HVA	78.4 ± 6.3
iso-HVA	97.9 ± 3.0

turbing superposition of the peaks of the catecholamines, MHPG, NMN, HVA, 5-HT, iso-HVA and 5-H-NMetT, is abolished owing to the separate extraction of the catecholamines and the phenolic acids and alcohols by the combined pro-

TABLE II

RECOVERIES FOR NE, E, DA AND DHBA AFTER ION-PAIR EXTRACTION WITH TOABr

Compound	Recovery (mean ± S.D., $n = 10$) (%)
NE	100.9 ± 1.3
E	101.2 ± 1.6
DHBA	97.0 ± 1.7
DA	96.9 ± 1.4

TABLE III

OVERALL RECOVERIES FOR HUMAN CEREBELLUM SPIKED WITH ALL THE COMPOUNDS UNDER STUDY

Obtained by direct injection of the residual supernatant after consecutive ethyl acetate and ion-pair extraction with TOABr; recoveries are corrected for the different dilution steps due to the extraction procedure and for the concentrations measured in the blank; N.d. = not detected.

Compound	Recovery (mean ± S.D., $n = 8$) (%)
MHPG	50.1 ± 1.4
DOPAC	N.d.
5-HIAA	46.2 ± 2.1
HVA	19.4 ± 3.1
iso-HVA	N.d.
NE	N.d.
E	N.d.
DHBA	N.d.
DA	N.d.
NMN	96.3 ± 1.3
MN	89.7 ± 2.4
MHBA	91.6 ± 1.9
3-MT	87.8 ± 1.8
5-HT	89.9 ± 2.1
5-H-NMetT	89.4 ± 3.3

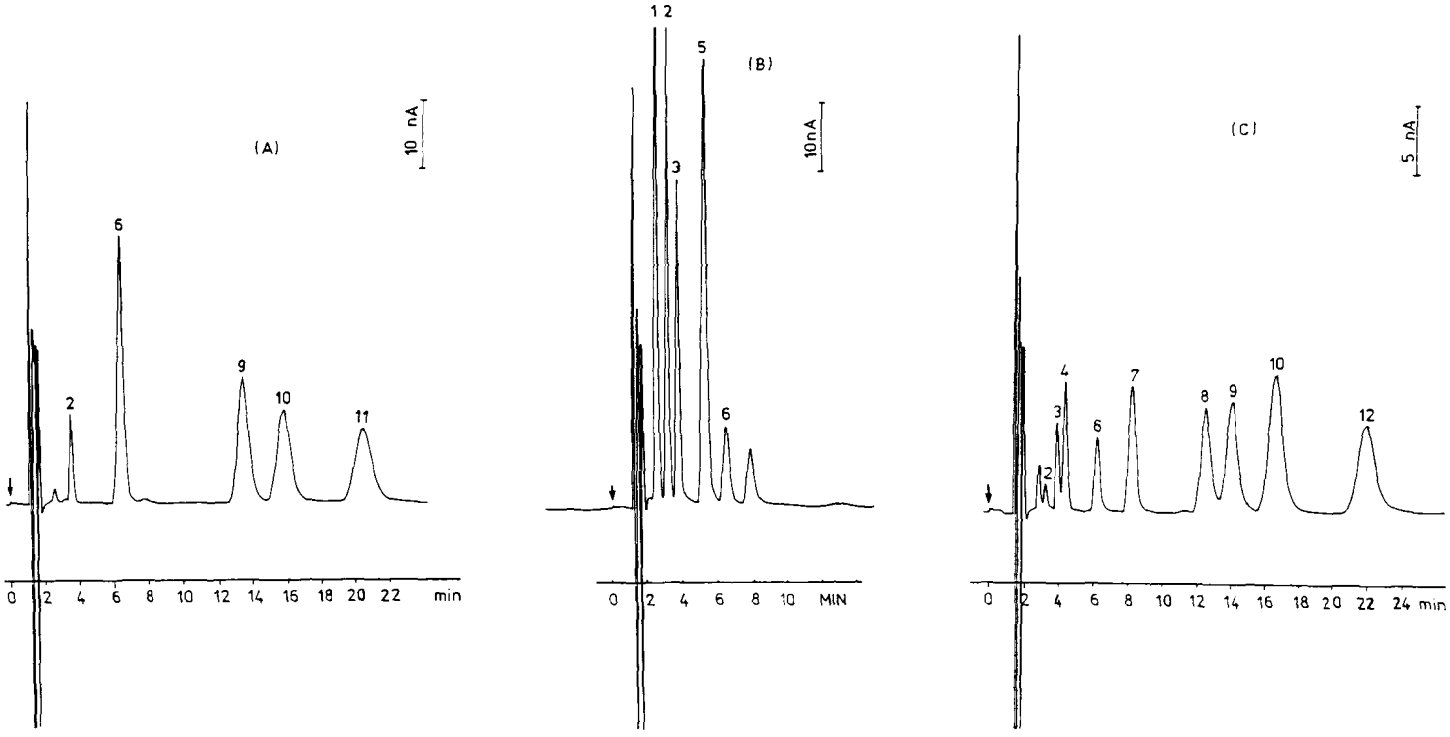


Fig. 3. (A) Chromatogram of 100 μl of the ethyl acetate extraction of homogenized human cerebellum spiked with a mixture of 100 ng/ml of all the compounds of interest. Peaks: 2 = MHPG; 6 = DOPAC; 9 = 5-HIAA; 10 = HVA; 11 = iso-HVA. Mobile phase, acetate–citrate buffer containing 1 m*M* 1-octanesulphonic acid at pH 3.0, methanol 5%; flow-rate, 1 ml/min; electrochemical detection at +0.75 V, range 50 nA. (B) Chromatogram of 100 μl of the catecholamine ion-pair extraction with TOABr in the presence of diphenylborate of the same mixture. Peaks: 1 = NE; 2 = E; 3 = DHBA; 5 = DA; 6 = DOPAC. Mobile phase, acetate–citrate buffer containing 1 m*M* octanesulphonic acid at pH 3.0, methanol 1%; flow-rate and detection as in A. (C) Chromatogram of 100 μl of the residual supernatant after the two consecutive extractions (A + B). Peaks: 1 = MHPG; 2 = NMN; 3 = MN; 4 = DOPAC; 5 = MHBA; 6 = 3-MT; 7 = 5-HIAA; 8 = 5-HT; 9 = 5-H-NMetT. Mobile phase and flow-rate as in A; detection at +0.75 V, range 20 nA.

TABLE IV

CONCENTRATIONS OF THE COMPOUNDS UNDER STUDY

Values in pmol/mg wet brain tissue obtained after application of the combined extraction procedure and HPLC-electrochemical detection on some human brain locations (mean values ± S.D. of four determinations).

Location	DA	3-MT	DOPAC	HVA	
Frontal cortex	0.06 ± 0.02	< 0.05	0.09 ± 0.03	0.95 ± 0.27	
Visual cortex	0.07 ± 0.02	< 0.05	0.12 ± 0.07	1.06 ± 0.28	
Cerebellum	0.05 ± 0.03	< 0.05	0.06 ± 0.02	0.97 ± 0.30	
Caudate nucleus	7.16 ± 2.54	4.31 ± 1.02	5.88 ± 2.31	39.05 ± 13.07	
Putamen	7.07 ± 4.44	4.36 ± 0.93	6.14 ± 0.43	50.74 ± 28.38	
Locus coeruleus	0.37 ± 0.14	1.04 ± 0.31	1.66 ± 0.93	12.31 ± 1.57	
	NE	E	NMN	MN	MHPG
Frontal cortex	0.24 ± 0.05	0.05 ± 0.01	0.14 ± 0.02	0.10 ± 0.01	0.23 ± 0.02
Visual cortex	0.09 ± 0.02	0.03 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.25 ± 0.05
Cerebellum	0.11 ± 0.04	0.02 ± 0.01	0.65 ± 0.17	< 0.04	0.29 ± 0.05
Caudate nucleus	0.10 ± 0.03	0.06 ± 0.01	1.25 ± 0.35	1.03 ± 0.21	0.31 ± 0.05
Putamen	0.11 ± 0.02	0.05 ± 0.01	1.66 ± 0.41	1.22 ± 0.23	0.29 ± 0.06
Locus coeruleus	2.90 ± 0.81	1.44 ± 0.58	3.61 ± 0.52	2.87 ± 0.37	2.33 ± 0.11
	5-HT	5-HIAA			
Frontal cortex	1.68 ± 0.22	1.01 ± 0.36			
Visual cortex	1.51 ± 0.09	1.12 ± 0.19			
Cerebellum	0.50 ± 0.11	0.91 ± 0.29			
Caudate nucleus	3.79 ± 0.78	1.60 ± 0.24			
Putamen	1.97 ± 0.39	1.78 ± 0.49			
Locus coeruleus	2.04 ± 0.55	71.42 ± 13.38			

turbing superposition of the peaks of the catecholamines, MHPG, NMN, HVA, 5-HT, iso-HVA and 5-H-NMetT, is abolished owing to the separate extraction of the catecholamines and the phenolic acids and alcohols by the combined procedure. The between-day precision for all the compounds under study varies between 1.29 and 12.31% for eight determinations.

About 40 brain tissue samples can be treated by this combined technique during one working day. A disadvantage of this extraction system compared with the direct injection of the crude supernatant onto the HPLC system is the longer chromatography time required. The procedure can also be applied to the determination of the metanephrines and serotonin only. In this case the combined extraction is used as a clean-up procedure.

Application of the extraction scheme to brain tissue

To demonstrate the efficiency of these combined procedures, we performed a quantitative determination of all the compounds under study in some human brain tissue samples weighing from 10 to 100 mg wet weight. The regional distribution of all the monoamines and their metabolites is given in Table IV. Smaller

amounts (down to 0.5 pmol/g wet weight) can be detected if a concentration step is performed by reconstituting the acetic acid phase in smaller volumes in the ethyl acetate and ion-pair extraction steps, respectively. The sensitivity of the electrochemical detector can also be increased since the acetate–citrate mobile phase and low methanol concentrations used result in a good signal-to-noise ratio. The concentrations of the monoamines and their major metabolites measured are comparable with those previously reported (see refs. 20 and 21 for review). 3-MT concentrations are high owing to the time elapsed between death and obduction (4–6 h). DA is post-mortem metabolized in 3-MT owing to the remaining activity of the catechol-O-methyl transferase enzyme [22].

CONCLUSION

The proposed combined HPLC procedure permits a very sensitive and simultaneous quantitative determination of a great number of biogenic amines and their major metabolites in one single brain tissue sample of 10–50 mg of wet weight, permitting more complete studies of the relation and the interaction between the different monoamine neurotransmitter systems and their metabolites.

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