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DETERMINATION OF THE BIOGENIC AMINES AND THEIR MAJOR METABOLITES IN SINGLE HUMAN BRAIN TISSUE SAMPLES USING A COMBINED EXTRACTION PROCEDURE AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A combined extraction system for the selective and quantitative isolation of the monoamines norepinephrine, epinephrine, dopamine, serotonin (5-hydroxytryptamine) and their metabolites 3-methoxy-4-hydroxyphenylethylene glycol, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid, homovanillic acid and 3-methoxytyramine from one single brain tissue sample is described. The extraction system is a combination of an ethyl acetate extraction for 3-methoxy-4-hydroxyphenylethylene glycol, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid and homovanillic acid, and two successive ion-pair extractions. In a first step, the catecholamines are quantitatively isolated by extracting with heptane—octanol (99:1) containing 0.25% tetraoctylammonium bromide as an ion-pairing agent in the presence of 0.2% diphenylborate. In a second step, 3-methoxytyramine and 5-hydroxytryptamine are isolated from the aqueous phase with di(2-ethylhexyl)phosphoric acid as counter-ion in chloroform. Dihydroxybenzylamine, isohomovanillic acid and 5-hydroxy-N-methyltryptamine are used as the internal standards.

INTRODUCTION

The advantages of the determination of the biogenic amines by reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection (ED) have been clearly demonstrated during the last ten years. Clas-

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sically, the catecholamines norepinephrine (NE), epinephrine (E) and dopamine (DA) are isolated by adsorption on alumina [1—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]. Serotonin (5-HT) and 3-methoxytyramine (3-MT) are generally isolated by ion-exchange chromatography [6—8]. All these techniques offer separate assays of catecholamines, indoleamines and their acidic and alcoholic metabolites. Few methods permit the simultaneous determination of the three monoamines and their metabolites from a single sample of brain tissue or biological fluid [9]. An increasing number of publications during later years also mention the quantitative determination of various monoamines and their metabolites in biological material by direct injection of the supernatant onto an HPLC column [10—14].

The present paper describes an extraction scheme for the simultaneous quantitation of catecholamines, indoleamines and their major metabolites in human brain tissue weighing 10—100 mg. The extraction scheme combines three different solvent extraction systems: the alcoholic metabolite MHPG and the acidic metabolites DOPAC, 5-HIAA and HVA are extracted with ethyl acetate, the catecholamines are isolated by ion-pair extraction with tetraoctylammonium bromide (TOABr), while 5-HT and 3-MT are isolated with the ion-pairing agent di(2-ethylhexyl)phosphoric acid (DEHPA).

EXPERIMENTAL

Materials

The HPLC system consisted of a Varian 8500 (Varian, Walnut Creek, CA, U.S.A.) pulse-free pump equipped with a 100-μl Valco injection loop (Valco, Houston, TX, U.S.A.). The amperometric detector was a Bruker E 230 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). The mobile phase was degassed in a Branson-B 32 ultrasonic bath. 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 Hibar LiChrosorb RP-18 column (particle size 5 μm; 25 cm × 0.4 cm I.D.). The pre-column (3 cm × 0.4 cm I.D.) was packed with LiChrosorb RP-18 (particle size 10 μm), all from Merck (Darmstadt, F.R.G.).

Chemicals and reagents

Dopamine · HCl was purchased from UCB (Brussels, Belgium). *l*-Norepinephrine, *l*-epinephrine, homovanillic acid, tetraoctylammonium bromide and di(2-ethylhexyl)phosphoric acid were supplied by Fluka (Buchs, Switzerland). 3-Methoxy-4-hydroxyphenylethylene glycol piperazine, 3-methoxytyramine · HCl, serotonin · HCl hemihydrate, 5-hydroxyindoleacetic acid, 3,4-dihydroxybenzylamine · HBr (DHBA), 5-hydroxy-N-methyltryptamine oxalate (5-H-NMetT), diphenylborate—ethanolamine (DPBEA), di-*n*-butylamine and 1-octane sulphonic acid sodium salt were purchased from Janssen Chimica (Beerse, Belgium)/Aldrich-Europe. 3,4-Dihydroxyphenylacetic acid,

d,l-normetanephrine · HCl (NMN) and *d,l*-metanephrine · HCl (MN) were supplied by Sigma (St. Louis, MO, U.S.A.) and isohomovanillic acid (iso-HVA) was generously supplied by Roche (Basel, Switzerland). All other chemicals used were purchased from Merck. Deionized water was used throughout. Stock solutions of 10 mg per 100 ml were prepared by dilution of the standards in 0.01 *M* hydrochloric acid containing 0.1% $Na_2S_2O_5$ (w/v) and 0.01% EDTA (w/v) and stored at 4°C. Further dilutions were made in 0.5 *M* acetic acid.

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-octane sulphonic acid, 0.1 m*M* Na_2EDTA and 1 m*M* dibutylamine. The pH of the mobile phase was adjusted with concentrated phosphoric acid and the methanol concentration was chosen to achieve the shortest analysis time while keeping a good resolution of the monoamines and metabolites of interest. The flow-rate was set at 1 ml/min and the detector potential was +0.7 V versus the calomel reference electrode (range 50 nA).

RESULTS AND DISCUSSION

Optimization of the extraction procedure

Before combining the two ion-pair extraction techniques and the ethyl acetate extraction, each extraction procedure and the chromatographic conditions were optimized separately.

Extraction of the catecholamines NE, E, DA and DHBA. The ion-pair extraction technique used is an extension of the technique described by Smedes et al. for plasma and urine [15]. The extraction procedure is summarized in Scheme 1 (parts A and B) but here it was performed on aqueous standard solutions ranging from 10 to 200 ng/ml.

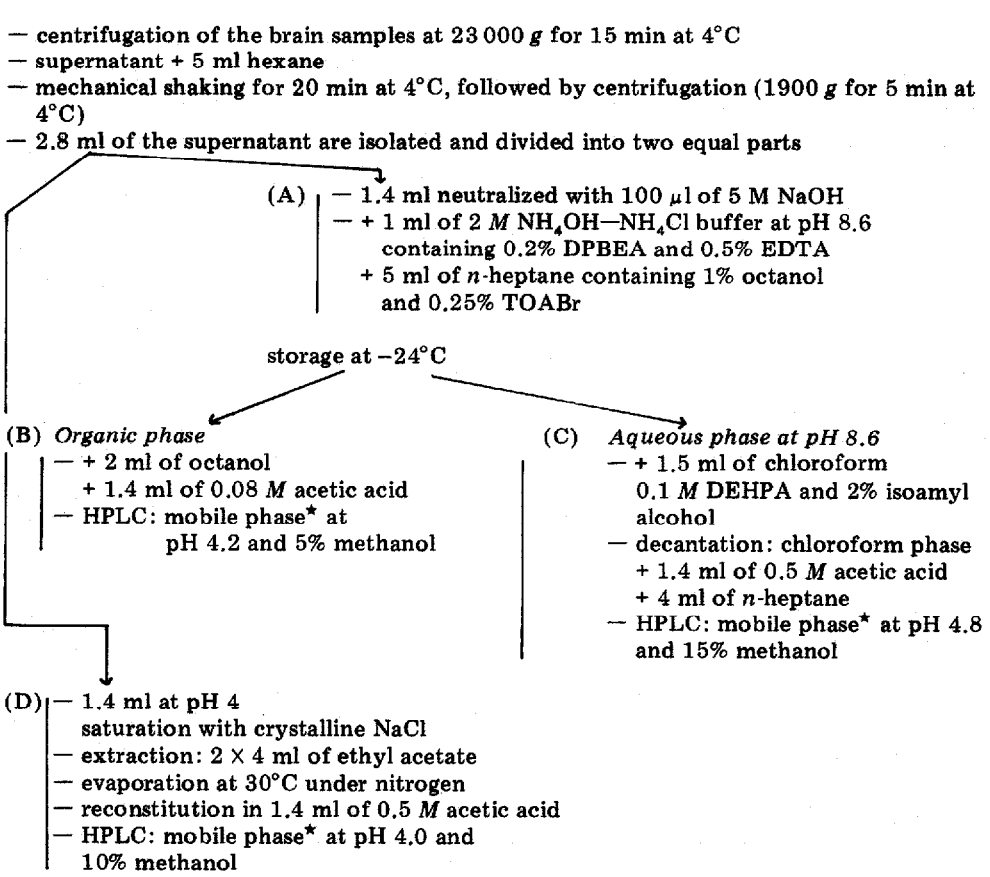
The percentage recoveries for NE, E, DA and DHBA are given in Table I. Quantitation was performed by injecting 100 µl of the acetic acid phase into the chromatograph with a mobile phase containing the acetate—citrate buffer and 1 m*M* 1-octanesulphonic acid (as ion-pairing agent) at pH 4.2, and methanol (95:5).

High and reproducible recoveries were obtained, and the results agreed with those obtained by Smedes et al. [15] for plasma and urine. The recoveries of the ion-pair extraction are far superior to those obtained by adsorption on

TABLE I

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

Compound	Percentage recovery (mean ± S.D., n = 5)
NE	101.0 ± 1.4
E	101.4 ± 2.2
DHBA	96.2 ± 1.5
DA	96.4 ± 1.6



*Mobile phase: acetate—citrate buffer containing 1 m*M* 1-octanesulphonic acid. pH and methanol concentrations are specified for each extraction.

Scheme 1. Schematic survey of the combined extraction procedure.

alumina [14, 16], ranging between 50 and 70% according to the method originally developed by Anton and Sayre [17, 18].

In a recent article, Kamperman and Kraak [19] mentioned that the pH of the ammonium hydroxide—ammonium chloride buffer should not exceed a value of 8, as at higher pH the methylated catecholamines NMN and MN, present in brain tissue, plasma and urine, could be partially converted into NE and E, resulting in an overestimation of the NE and E concentrations obtained after extraction with TOABr. We checked the finding of the authors by incubating a solution of NMN and MN in an ammonium hydroxide—ammonium chloride buffer at pH 8.6 for 1 h and comparing it with a mixture of NE, E, NMN and MN prepared as previously described. No conversion into NE or E could be detected after chromatographic analysis.

Extraction of 3-MT, 5-HT and 5-H-NMetT by ion-pair formation with DEHPA. 3-MT, 5-HT and 5-H-NMetT were extracted with di(2-ethylhexyl)-phosphoric acid (DEHPA) as the counter-ion. This procedure was first described in 1966 by Temple and Gillespie [20] for the extraction of physiolog-

ically active amines. Since then, only a few applications of this technique in the study of some monoamines in the central nervous system have been described [21—23]. In our laboratory, the use of DEHPA in the ion-pair extraction of basic drugs has been studied extensively [24, 25].

The extraction procedure is summarized in Scheme 1 (parts A and C) but here it was also performed on aqueous standard solutions ranging from 10 to 200 ng/ml. The influence of pH on the recovery was studied first. Fig. 1 gives the recovery as a function of pH; each point is the mean of triplicate determinations. The HPLC conditions were as follows: the mobile phase consisted of an acetate—citrate buffer with 1 m*M* 1-octanesulphonic acid at pH 4.8 and 15% methanol (v/v). At this pH, the best chromatographic separation of 3-MT, 5-HT and 5-H-NMetT was obtained.

Fig. 1 shows that extraction at pH 5.2 yields the best recovery for 3-MT (70.3%), as found in the literature [22], but it is not very efficient for extracting 5-HT and 5-H-NMetT (mean values of 13.8 and 11.4%, respectively). The best recovery for 5-HT and 5-H-NMetT was obtained between pH 8 and 9 (mean values of 67.7 and 63.4%, respectively). The mean recovery of 3-MT at this alkaline pH was 20.6%.

Extraction of MHPG, DOPAC, 5-HIAA, HVA and iso-HVA with ethyl acetate. The extraction procedure is summarized in Scheme 1 (part D). The influence of the aqueous solution pH on the recovery was studied and the results are given in Fig. 2; each point is the mean of five determinations. The mobile phase of the HPLC system consisted of an acetate—citrate buffer with 1 m*M* 1-octanesulphonic acid at pH 4.0 and 10% methanol (v/v). At this pH, an

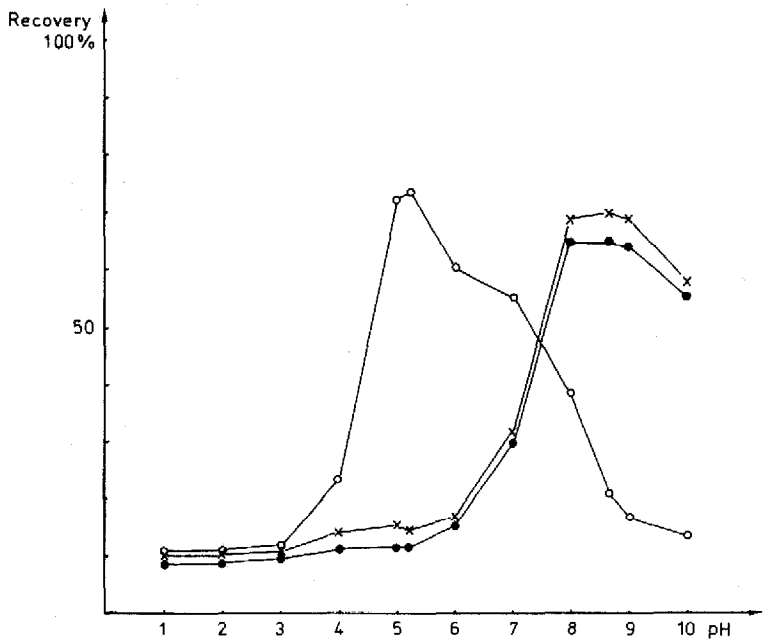


Fig. 1. Percentage recovery of the extraction of 3-MT (○), 5-HT (×) and 5-H-NMetT (•) as a function of pH (mean value of five determinations). The pH of the samples was adjusted with a 1 *M* phosphate buffer ranging from pH 1 to 10.

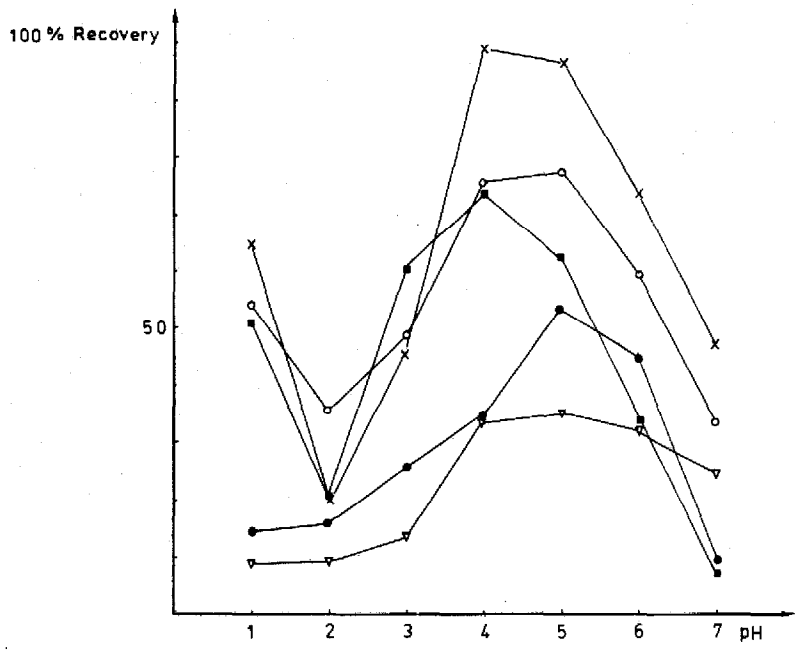


Fig. 2. Percentage recovery of the extraction of MHPG (▽), DOPAC (■), 5-HIAA (●), HVA (○) and iso-HVA (×) as a function of pH (without salting out).

excellent separation of all the neutral and acidic compounds under study was obtained.

A satisfactory recovery for MHPG, DOPAC, 5-HIAA, HVA and iso-HVA was found at pH 4. This assay yields a relatively low recovery at pH 1 for DOPAC, HVA and iso-HVA, the three metabolites that are usually extracted at this pH. Saturation of the samples with sodium chloride does not improve the extraction recovery of iso-HVA at both pH values studied, but yields a higher recovery for DOPAC and HVA at pH 1 and for MHPG and 5-HIAA at pH 4. Therefore, the extraction was performed at pH 4 after salting out. The results are reported in Table II.

TABLE II

PERCENTAGE RECOVERY AT pH 1 AND 4 WITHOUT AND WITH SODIUM CHLORIDE SATURATION

Compound	Percentage recovery (mean ± S.D.)			
	pH 1		pH 4	
	Without NaCl (n = 5)	With NaCl (n = 3)	Without NaCl (n = 5)	With NaCl (n = 3)
MHPG	8.3 ± 2.3	12.3 ± 4.8	43.2 ± 7.2	75.3 ± 6.1
DOPAC	51.4 ± 8.9	103.5 ± 6.7	70.3 ± 4.4	85.8 ± 5.8
5-HIAA	13.8 ± 6.1	23.4 ± 5.3	43.9 ± 5.4	80.6 ± 4.3
HVA	53.3 ± 10.2	101.6 ± 4.1	76.1 ± 7.4	80.9 ± 4.9
iso-HVA	65.0 ± 5.8	66.6 ± 3.2	98.4 ± 3.2	93.8 ± 3.2

Combination of both ion-pair extractions and ethyl acetate extraction: recovery study

The three previously described extraction procedures were tested on brain tissue (cerebellar cortex) homogenate spiked with the reference compounds. MHPG, DOPAC, 5-HIAA, HVA, NE, E, DA, 3-MT and 5-HT were quantitatively analysed with iso-HVA, DHBA and 5-H-NMetT [26] as internal standards.

Standard mixtures were diluted to 20, 100, 200, 300 and 400 ng/ml as previously described. A pool of 900 mg of cerebellum was homogenized in 5 ml of 1 *M* hydrochloric acid containing 0.1% $Na_2S_2O_5$ and 0.01% EDTA, and 5 ml of deionized water were added. From this suspension, six 1.3-ml aliquots were pipetted and added to 1.5-ml portions of each standard solution, together with 200 μl of an internal standard solution containing iso-HVA, DHBA or 5-H-NMetT at 1.5 g/ml. Iso-HVA is used as an internal standard for the ethyl acetate extraction, DHBA and 5-H-NMetT as internal standards for the TOABr and the DEHPA extraction, respectively. A 1.3-ml volume of the same cerebellum suspension was used as a blank. No compounds under study are expected to be detected in this sample owing to the dilution factor (reconstitution of the samples in 1.4 ml of 0.5 *M* acetic acid).

Scheme 1 summarizes the extraction and the overall recoveries of the combined extraction procedure are given in Table III.

No significant difference in recovery could be found for the extraction of MHPG, DOPAC, 5-HIAA, HVA and iso-HVA with ethyl acetate compared to the single procedure. The same conclusion can be drawn for the catecholamines extracted with TOABr. However, when we compared the results obtained for the extraction of 3-MT, 5-HT and 5-H-NMetT from brain tissue with the single procedure performed on aqueous standards, we found a lower recovery (see Fig. 1 and Table III). This same phenomenon has also been described in the literature [27—29]. The origin of this low recovery was not studied, but possi-

TABLE III

PERCENTAGE OVERALL RECOVERY OF THE COMBINED EXTRACTION SCHEME

Compound	Percentage overall recovery (mean ± S.D., n = 5)
MHPG	70.0 ± 5.3
DOPAC	81.4 ± 4.8
5-HIAA	81.3 ± 3.9
HVA	82.9 ± 4.7
iso-HVA	97.8 ± 2.9
NE	101.2 ± 1.0
E	101.3 ± 0.7
DHBA	97.5 ± 1.8
DA	96.8 ± 1.7
3-MT	6.1 ± 0.5
5-HT	44.8 ± 2.9
5-H-NMetT	39.1 ± 2.0

ble causes can be the presence of lipids and emulsification during mechanical shaking, as well as the formation of ternary complexes between monoamines, counter-ion and residual brain tissue proteins. Iso-HVA, DHBA and 5-H-NMetT are very suitable as internal standards. Each of them is selectively extracted (97.8 ± 2.9%, 96.8 ± 1.7% and 39.1 ± 2.0%, respectively; mean ± S.D. of six determinations) and the calibration graphs are linear in the range 1—20 ng on the column.

Application of the extraction scheme to brain tissue

A great advantage of this combined extraction technique is the possibility of determining the monoamines and their metabolites in one single brain area of

TABLE IV

CONCENTRATIONS OF THE COMPOUNDS UNDER STUDY OBTAINED AFTER APPLICATION OF THE COMBINED EXTRACTION PROCEDURE AND HPLC—ED ON HUMAN BRAIN TISSUE LOCALIZATIONS

Location	Sample weight (mg)	Concentration (ng/g of wet brain tissue)								
		DA	DOPAC	HVA	NAD	AD	MHPG	5-HT	5-HIAA	3-MT
Frontal cortex	164.2	15.4	11.6	147.9	41.4	9.1	64.9	403.2	76.6	
Cerebellum	176.4	12.6	7.0	220.1	12.8	5.6	79.6	92.1	59.3	
Caudate nucleus	37.8	1008.3	791.2	5843.2	20.5	12.8	204.5	459.1	264.4	516.0
Corpus mammillare	22.8	56.6	219.9	1178.3	354.6	25.7	1502.0	117.5	587.9	
Locus coeruleus	19.7	51.7	168.0	2041.6	577.4	241.6	1414.7	3114.1	11846.2	

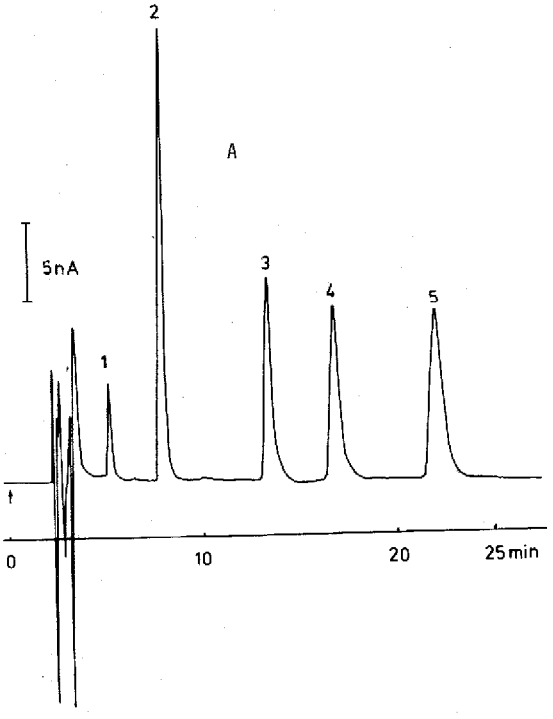


Fig. 3.

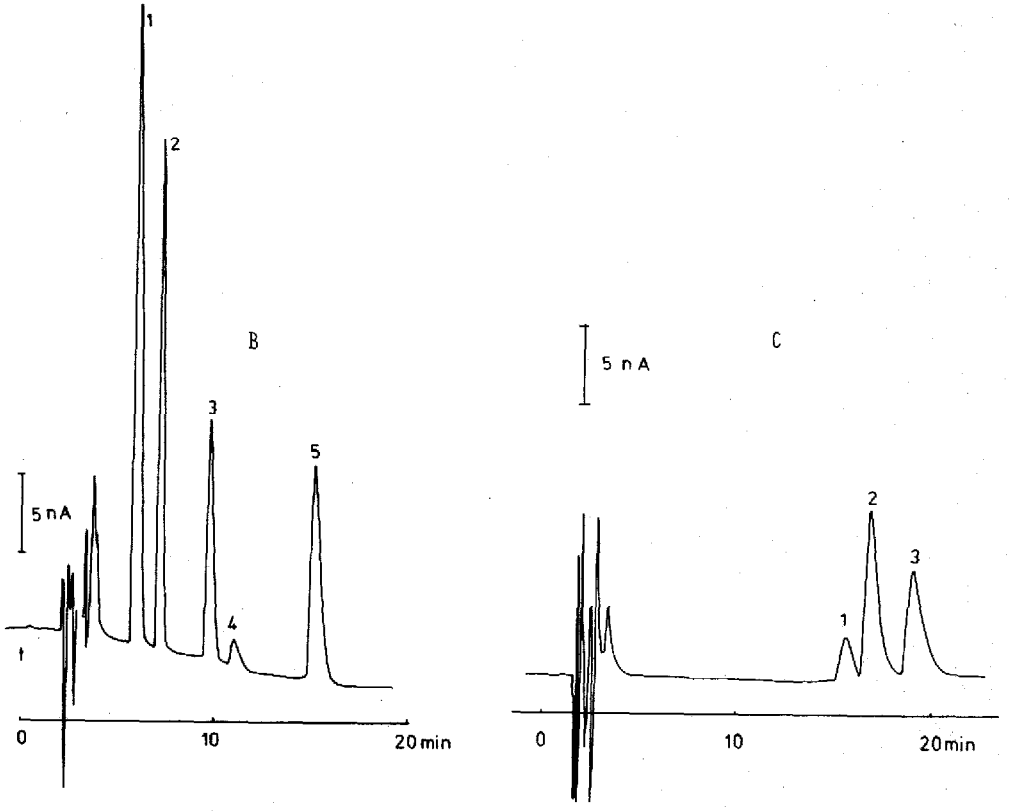


Fig. 3. Chromatograms of brain tissue spiked with standards. (A) Ethyl acetate extraction; peaks: 1 = MHPG; 2 = DOPAC; 3 = 5-HIAA; (B) Extraction with TOABr; peaks: 1 = NAD; 2 = AD; 3 = DHBA; 4 = DOPAC; 5 = DA. (C) Extraction with DEHPA at pH 8.6; peaks: 1 = 3-MT; 2 = 5-HT; 3 = 5-H-NMetT.

10—100 mg of wet weight. Concentration of the samples is possible by back-extraction of the monoamines in a smaller volume of acetic acid compared to the original sample. By this means, very low quantities of compounds (up to 10 ng/g of wet brain tissue), such as those found in cortical regions, can easily be quantified.

As an example, some results of a study of the regional distribution of monoamines and their metabolites in normal human brain tissue are given in Table IV.

The number of samples that can be treated by this combined extraction technique during one working day varies between 35 and 45. Another advantage of the proposed procedure on the direct injection of supernatant after centrifugation of homogenized brain tissue is the simplicity of the chromatograms obtained, due to the large selectivity of the different extraction procedures (see Fig. 3).

Reconstituted aliquots injected in the chromatograph are very clean, permitting up to 800 injections on a single analytical column combined with a pre-column without any signs of alterations.

CONCLUSION

The technique described allows a reliable and precise determination of the four monoamines and their metabolites in small brain tissue samples.

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