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QUANTIFICATION OF A NUMBER OF BIOGENIC AMINES AND THEIR METABOLITES IN BRAIN HOMOGENATES BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A sensitive method is described for the assay of various biogenic amines and their metabolites from brain tissue homogenates. Based on gas chromatography—mass spectrometry and selected ion monitoring, the method allows reliable low-level quantification of the compounds of interest. By careful study of reaction conditions both in the preparation of derivatives for gas chromatography and in the synthesis of deuterated analogues for internal standard use, relatively simple procedures could be applied with good analytical efficiency. Correlation of the results with those obtained by a high-performance liquid chromatographic procedure was also investigated, and a neurologic application of the method is briefly discussed.

INTRODUCTION

Various neurologic and psychiatric diseases seem to be characterized by alterations in brain levels of adrenaline, noradrenaline, dopamine, serotonin, 3-methoxy-4-hydroxyphenylethylene glycol, 3,4-dihydroxyphenylacetic acid, homovanillic acid and 5-hydroxy-3-indoleacetic acid [1—3]. Reliable quantification of the extremely low levels of such compounds in brain tissue necessitates application of sophisticated techniques, such as gas chromatography—mass spectrometry (GC—MS) [4], or electrochemical detection of compounds separated by high-performance liquid chromatography (HPLC) [5].

Using deuterated analogues as the corresponding internal standards, selected ion monitoring (SIM) provides an ideal basis for specific quantitative analysis by GC—MS, especially in association with quadrupole instruments [6, 7].

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The polar character of the compounds studied here — catecholamines, serotonin, and the four metabolites — necessitates their derivatization prior to gas chromatography. Several methods are available for reactions involving phenolic and alcoholic hydroxyl groups and primary or secondary amino groups present in these molecules [4, 8–13]. In the present investigation, acylation with pentafluoropropionic anhydride and esterification with pentafluoropropanol were used for derivatization, optimum reaction conditions being found by systematic selection and modification of appropriate methods.

Deuterium labelling was also investigated in detail, and relatively mild reaction conditions are described for an efficient conversion of the compounds of interest to the corresponding deuterated analogues by electrophilic substitution of the aromatic ring hydrogens [14–16].

EXPERIMENTAL

Chemicals used in the present investigation were obtained from the following sources: 3,4-dihydroxyphenylacetic acid (DOPAC), (—)-adrenaline (+)-bitartrate, 3-methoxy-4-hydroxy-phenethanol (MOPET), (—)-noradrenaline bitartrate, 3-hydroxytyramine (dopamine) hydrochloride and 3,4-dihydroxybenzylamine (DHBA) from Sigma (St. Louis, MO, U.S.A.); homovanillic acid (HVA), serotonin monooxalate, potassium salt of 3-methoxy-4-sulfonyloxyphenylethyleneglycol (MHPG) and 5-hydroxy-3-indoleacetic acid (5-HIAA) from Fluka (Buchs, Switzerland); 2,2,3,3,3-pentafluoropropanol from Koch-Light Labs. (Colnbrook, U.K.); acetic-d_3- acid-*d* (99.5 atom %) and deuterium oxide (100 atom %) from Aldrich (Milwaukee, WI, U.S.A.); deuterium chloride (20% solution in ^2H_2O) and sulphuric acid-*d* (98% solution in ^2H_2O) from EGA Chemie (Steinheim, F.R.G.); pentafluoropropionic anhydride (PFPA) from Pierce (Rockford, IL, U.S.A.); boron trichloride methanol (BCl_3—methanol, 10% w/v) from Applied Science Labs. (State College, PA, U.S.A.); Amberlite-XAD type 2 resin, particle size 0.3—1.0 mm from Serva Feinbiochemica (Heidelberg, F.R.G.). All other chemicals and solvents used were obtained from E. Merck (Darmstadt, F.R.G.).

Homogenization of brain tissue

Samples (0.8—1.0 g) of brain tissue were suspended in distilled water (1:3) containing 0.1% of $Na_2S_2O_5$ and 0.01% of sodium EDTA, and 400—700 μl of this homogenate were used for the assay. The pH was adjusted to 1—2 with a solution of 0.1 *M* hydrochloric acid, and 25 ng or 50 ng of appropriate internal standards were added. The suspension was finally vortex-mixed and centrifuged at 15,000 *g* for 20 min.

Extraction of adrenaline, noradrenaline and dopamine

The supernatant from homogenization (as above), 50 mg of alumina (Al_2O_3), and 2.5 ml of 0.5 *M* Tris buffer (pH 8.6) were mixed in a test tube. After centrifugation, the supernatant was removed and the alumina was washed with 10 $\times$ 1 ml of distilled water. The catecholamines were extracted from the alumina with 2 $\times$ 0.4 ml of a solution of 0.4 *M* acetic acid in methanol (1:45). After solvent evaporation with a stream of nitrogen, the residue was treated as described under Derivatization (see below).

Extraction of serotonin, DOPAC, HVA, MHPG and 5-HIAA

Either 25 ng or 50 ng of the appropriate internal standards were added to the supernatant from homogenization and this solution was slowly applied to a column (0.8 × 2 cm) containing Amberlite XAD-2 resin. After washing with 15 ml of diluted hydrochloric acid, the column was eluted with 3 × 0.7 ml of methanol. The eluate was evaporated to dryness under a stream of nitrogen and the residue was treated as described under derivatization. The extraction system was found to be quantitative in experiments using authentic compounds.

Derivatization

Standard solutions of adrenaline, noradrenaline, dopamine and serotonin (10 μg in 0.1 ml of methanol) were evaporated to dryness in test tubes under a stream of nitrogen. Then 0.1 ml of BCl_3—methanol was added and the tubes were maintained at 45°C for 15 min. After solvent evaporation, 0.1 ml of PFPA was added, and the reaction was allowed to proceed at 45°C for 1.5 h. The reagent was removed with a stream of nitrogen and the residue dissolved in 50 μl of ethyl acetate. The same procedure was used for the appropriate extraction residues (see above) and deuterated compounds (see below), while the metabolites DOPAC, HVA, MHPG and 5-HIAA, the corresponding extraction residues and deuterated compounds were derivatized according to the procedure reported in ref. 13, but using double reagent volumes.

Preparation of deuterated compounds for use as internal standards in SIM

Dopamine. 10 mg were dissolved in 2 ml of a solution of 20% ^2HCl in ^2H_2O, and maintained at 130°C for 21 h in a stoppered test tube. The product was recrystallized from methanol—diethyl ether (1:1) and stored at —20°C under nitrogen [14, 15].

Serotonin. 10 mg were dissolved in 2 ml of a solution of 20% $^2H_2SO_4$ in ^2H_2O and purged with nitrogen for 2 h. Aliquots (1 ml) of the solution were maintained at 45°C for 50 h or 72 h in stoppered test tubes. After cooling to room temperature, 150 mg of ascorbic acid were added to the solutions, which were then adjusted to pH 10.0 by dropwise addition of 2 *M* NH_4OH. Extraction with 3 × 2 ml of ethyl acetate* yielded stock solutions which were stored in stoppered test tubes under nitrogen at —20°C.

HVA. 30 mg were dissolved in a solution prepared by mixing 3.8 ml of 20% ^2HCl in ^2H_2O, 0.5 ml of $C^2H_3COO^2H$ and 1 ml of ^2H_2O. The solution was refluxed at 190°C for 30 min, with a stream of nitrogen directed into the cooler. After cooling to room temperature, the solution was extracted with 3 × 5 ml of ethyl acetate. The solvent was evaporated with a stream of nitrogen and the product recrystallized from 2 ml of benzene—diethyl ether (1:1). The crystals obtained were stored under nitrogen at —20°C.

DOPAC. 15 mg were dissolved in 0.7 ml of a soluton of 20% ^2HCl in ^2H_2O and incubated at 60°C for 5 h in a stoppered test tube. The solution was extracted with 3 × 4 ml of ethyl acetate. Evaporation of the solvent under a stream of nitrogen yielded a crystalline residue which was stored under nitrogen at —20°C.

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*All ethyl acetate extractions were performed with freshly distilled, nitrogen-purged solvent.

5-HIAA. 5 mg was dissolved in 2 ml of a 10% solution of $^2H_2SO_4$ in ^2H_2O. The solution was purged with nitrogen for 2 h and divided into three aliquots which were incubated in stoppered test tubes under nitrogen at 50°C for 21, 50 and 72 h, respectively. After cooling to room temperature, 50 mg of ascorbic acid were added to each of the solutions, which were then extracted with 3 × 5 ml of ethyl acetate. The solutions obtained were stored under nitrogen at −20°C.

MHPG. Attempts to prepare deuterated analogues of this compound were unsuccessful.

Derivatization of deuterated compounds

The deuterated amines and metabolites were derivatized for gas chromatography according to the procedures described under Derivatization except in the case of 5-HIAA. The deuterated analogue of this compound was acylated with PFPA at 45°C instead of 75°C.

Gas chromatography—mass spectrometry

A Hewlett-Packard 5990 A quadrupole GC—MS system (with HP 9825 B calculator and HP 9876 A printer) was used. The gas chromatograph was equipped with an SE-30 fused-silica capillary column (25 m × 0.32 mm I.D., Orion Analytica). Helium was used as carrier gas with a flow-rate of 3 ml/min. The GC injector and the GC/MS interface were maintained at 220°C and 210°C, respectively. The column oven was maintained at 70°C during injection and raised to 90°C immediately after elution of the solvent peak. After 1 min, the temperature was increased by 10°C/min to 170°C, and this temperature was maintained until the analysis was completed. The electron impact source of the mass spectrometer was operated at 70 eV, and the detector multiplier voltage was 2600 V.

TABLE I

RESULTS FROM THE PREPARATION OF DEUTERATED ANALOGUES (INTERNAL STANDARDS)

Compound	MW*	Incubation conditions		Relative (%) yields of products**						Ion (m/e)
		Temperature (°C)	Time (h)	d_0	d_1	d_2	d_3	d_4	d_5	
Adrenaline	753	45	4	59	100	59	16	3	—	590
Adrenaline		45	27	—	23	91	100	18	2	590
Noradrenaline	767	45	4	54	100	68	19	3	—	445
Noradrenaline		45	16	3	34	90	100	17	—	590
Noradrenaline		45	38 (10% ^{2}HCl)	41	100	60	27	3	—	445
Dopamine	591	130	21	—	—	7	100	—	—	428
Serotonin	614	45	50	76	100	49	14	1	—	451
DOPAC	592	60	5	1	27	100	15	2	—	415
5-HIAA	615	50	21	40	71	100	55	11	—	438
5-HIAA		40	50	30	88	100	42	9	1	438
HVA	460	190	0.5	—	—	4	100	—	—	460

*MW = molecular weight.

**The values are only approximate since isotope effect may influence fragmentation.

High-performance liquid chromatography

The procedures and solvent systems described in ref. 5 were directly applied to the homogenate extracts (see above). The HPLC equipment consisted of an Altex 110 A pump, an Altex Ultrasphere ODS (C_{18}) column (25 × 0.4 cm, particle size 5 μm), a BAS LC 4 B amperometric detector with glassy carbon electrode, and a Shimadzu C-R1A Chromatopac integrator. Detector voltages of +0.60 V and +0.85 V were used in the analysis of amines and metabolites, respectively. The internal standard for amines was DHBA (0.2 μM), while MOPET (1.0 μM) was used as internal standard in the quantification of metabolites. The sample loop volume was 20 μl.

RESULTS AND DISCUSSION

Extraction of the compounds studied yielded consistent results; however, in the case of serotonin the procedure was unsatisfactory.

Esterification of the carboxyl groups of a number of catecholamine metabolites using BCl_3—methanol as the reagent has been previously reported [16,

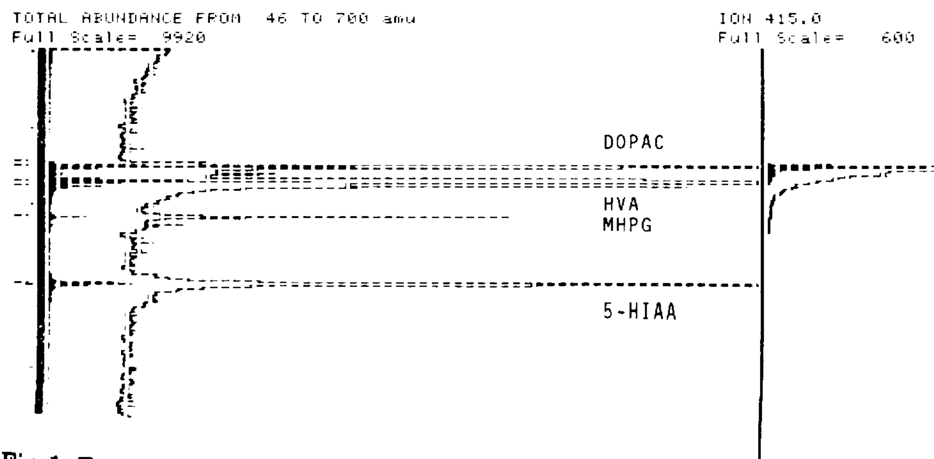

Fig. 1. Total ion current chromatogram showing the separation of derivatized DOPAC, HVA, 5-HIAA and MHPG.

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6-ION SIM DATA PROCESSOR, (Rev. 12/9/77)

*** SAMPLE IDENTITY #          1.00 dated   5/10/1983  Tuesday

ION MASSES              190.00  445.00  428.00  451.00
DWELL TIMES (msec)       50.00   50.00   50.00   50.00
MAXIMUM ABUNDANCE       347.84  116.40  174.31    6.66

RUN 2   TOTAL RUN TIME = 17.0        AMOUNT INJECTED =    1.00
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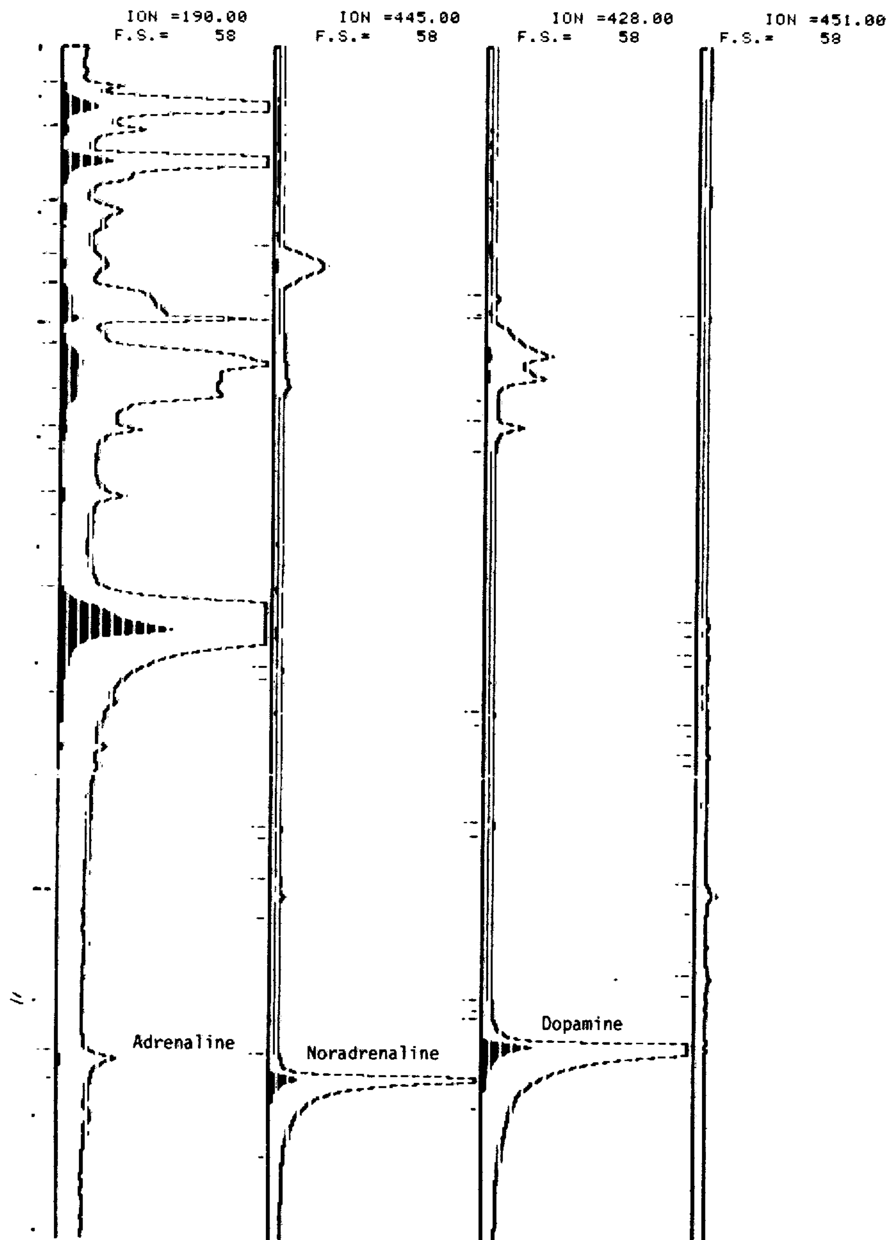


Fig. 2. SIM chromatogram traces for catecholamines from brain homogenate. Retention times (min): dopamine 11.3, adrenaline 11.4, noradrenaline 11.7 (uppermost trace is tuned for serotonin not present in the sample).

17]. In the present investigation, BCl_3—methanol was introduced as catalyst in the PFPA-acylation of amines. The reactions of all four amines studied proceeded smoothly and quantitatively at 45°C. A reaction temperature of 75°C, required for acceptable acylation rates in the absence of BCl_3, invariably led to partial decomposition of noradrenaline, as evidenced by MS.

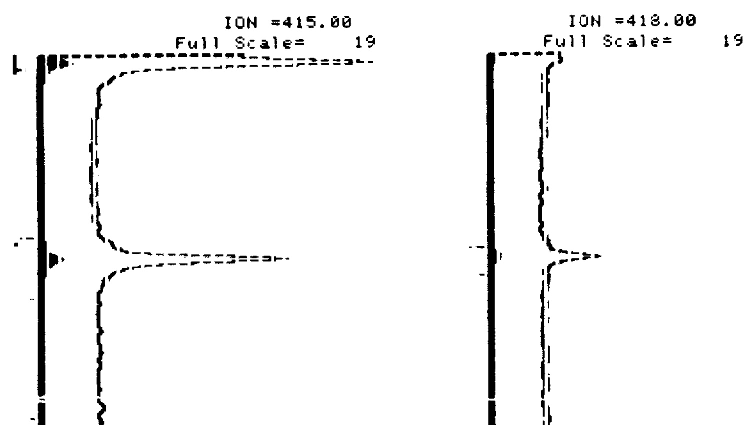
Mass spectrometric fragmentation of the derivatized compounds, as well as appropriate ions for SIM have been dealt with in refs. 13 and 16. In general, the compounds studied are prone to oxidative degradation during acid-catalysed synthesis of deuterated analogues. However, the simple procedures

6-ION SIM DATA PROCESSOR, (Rev. 12/9/77)

*** SAMPLE IDENTITY # 3.00 dated 5/23/1983 Monday

ION MASSES	415.00	418.00
DWELL TIMES (msec)	50.00	50.00
MAXIMUM ABUNDANCE	19.28	5.52

RUN 4 TOTAL RUN TIME = 7.9 AMOUNT INJECTED = 1.00



INTEGRATION SENSITIVITY =0.040 AREA THRESHOLD = 10 SMOOTHING FACTOR =0.80

1 ION 415.00	PEAK	RET. TIME	AREA	AREA/(SUM OF AREAS)	AREA/(LARGEST PEAK)
	1	7.1	531.8	97.24936	100.00000
	2	7.8	15.0	2.75064	2.82844
SUM OF AREAS =			546.8		

2 ION 418.00	PEAK	RET. TIME	AREA	AREA/(SUM OF AREAS)	AREA/(LARGEST PEAK)
	1	7.1	113.0	100.00000	100.00000
SUM OF AREAS =			113.0		

+++++++++++++ DATA NORMALIZED ON AREA + 113.0

1 ION 415.00	PEAK	RET. TIME	AREA	% AREA
	1	7.1	531.8	470.58577
	2	7.8	15.0	13.31023

2 ION 418.00	PEAK	RET. TIME	AREA	% AREA
	1	7.1	113.0	100.03622

Fig. 3. SIM quantification of DOPAC from brain homogenate. Amount of internal standard 500 pg/µl (*m/e* 418), peak height ratio 4.71, calculated amount of DOPAC 969 pg/µl (*m/e* 415).

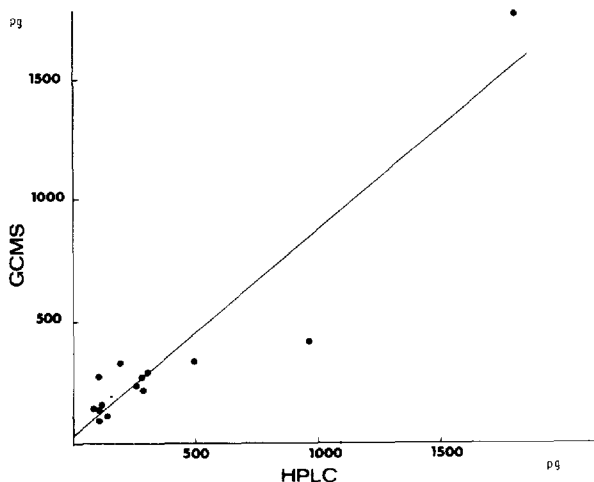


Fig. 4. Correlation of results obtained in the quantification of 5-HIAA by GC—MS and HPLC. Regression equation $Y = 0.83X + 27$, coefficient of correlation $r = 0.940$, $n = 14$.

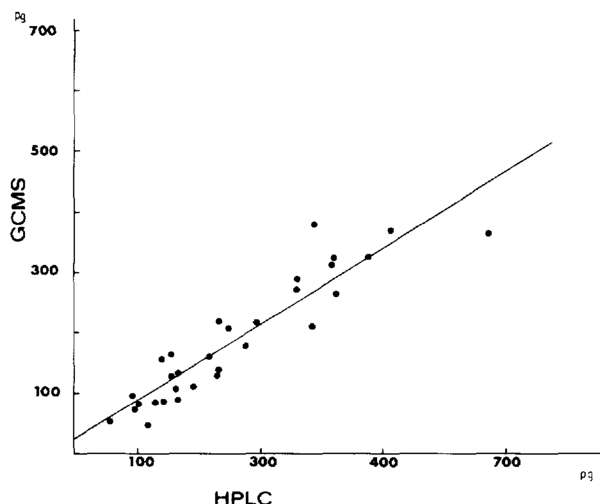


Fig. 5. Correlation of results obtained in the quantification of HVA by GC—MS and HPLC. Regression equation $Y = 0.62X + 25$, coefficient of correlation $r = 0.920$, $n = 31$.

used here are entirely satisfactory. It is noteworthy that deuterated serotonin and 5-HIAA could be obtained in good yields by using relatively high antioxidant concentrations.

Evaluation of the degree of incorporation of deuterium atoms in the compounds was undertaken by SIM, and the results are presented in Table I. In the case of adrenaline, the evaluation was hampered by a low relative intensity of the ion at *m/e* 590. The ion at *m/e* 445 would seem to provide a sufficiently reliable basis for the evaluation of deuterium incorporation in noradrenaline under the conditions used.

Fig. 1 shows a GC test run for the separation of DOPAC, HVA, MHPG and 5-HIAA, while selected-ion chromatogram traces for catecholamines in a brain sample are shown in Fig. 2. Typical calibration data for DOPAC and dopamine yielded correlation coefficients of $r = 0.997$ and $r = 0.984$, respectively.

TABLE II

NORADRENALINE AND DOPAMINE LEVELS MEASURED BY GC—MS IN VARIOUS BRAIN AREAS OF HISTOLOGICALLY VERIFIED PATIENTS WITH ALZHEIMER'S DISEASE AND DEMENTIA, AND OF THE CORRESPONDING CONTROL GROUPS

	Noradrenaline		Dopamine	
	ng/g ± S.D.	*n*	ng/g ± S.D.	*n*
Frontal cortex	12 ± 2	2*	171 ± 127	2
Temporal cortex	<20	4	187 ± 30	4
Hippocampal cortex	<20	2	155 ± 37	3
Thalamus	173 ± 96	6	184 ± 103	8
Nucleus caudatus	76 ± 86	6	1788 ± 188	8
Putamen	340 ± 336	4	713 ± 816	4
Pons	98 ± 45	2	114 ± 70	2

*Larger homogenate volume than usual.

Fig. 3 shows SIM responses for DOPAC and the corresponding internal standard. Correlation between the two analytical methods used, GC—MS and HPLC—electrochemical detection is evidenced by regression data in Figs. 4 and 5 for 5-HIAA and HVA, respectively.

The precision of the SIM method is relatively good; for example, for DOPAC, the mean amount per g of brain tissue is 399.8 ± 56.3 ng, n = 5, range = 326—457 ng, C.V. = 14%.

Table II summarizes the results from quantification of noradrenaline and dopamine from various brain areas of histologically verified patients with Alzheimer's disease, dementia, and of the corresponding controls. The high values of standard deviations are indicative of the very different amine levels associated with this type of material.

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