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ISOLATION, SEPARATION AND ANALYSIS IN NEUROCHEMISTRY: TRACE AMINES AND ACIDS AS AN ILLUSTRATIVE EXAMPLE

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SUMMARY

In this brief overview various neurochemical isolation procedures that can be adopted for the analysis of several monoamine neurotransmitters/neuromodulators and their principal oxidatively deaminated metabolites are outlined. With respect to the trace amines, they can be identified and quantitated as their so-called dansyl derivatives after thin-layer chromatographic separation by mass spectrometric (MS) electron-impact (EI) ionisation followed by selected-ion monitoring (SIM) of their molecular ions. Deuterated homologues are added as internal standards at the start of the analytical procedure. The MS–EI–SIM procedure offers a tissue extract or releasate sensitivity of about 100 pg/g of tissue or fluid. In the case of tryptamine or phenylethylamine, by utilising different derivatives (N-acetylpentafluoropropionyl or N-acetylpentafluorobenzoyl), which cyclise to form perfluorinated spirocyclic compounds, it is possible using MS negative chemical ionisation techniques coupled with monitoring of the (M−HF) ions to achieve sensitivities for tissue extracts of 1 pg/g or less. Acidic and neutral metabolites (up to twelve of them can be assayed simultaneously) can be detected and quantitated in tissue extracts, releasates or biological fluids as their methylpentafluoropropionyl or trifluoroethyl-pentafluoropropionyl derivatives in the 100–1000 pg range using gas chromatographic–MS–SIM procedures.

INTRODUCTION

The brain is unlike any other organ, being heterogeneous and containing in the main two distinct cell types: neurones and glia. The former number about ten billion (10^{10}) and it has been suggested that their synaptic connections could number ten trillion (10^{13}). The number of putative neurotransmitters is thought to exceed thirty [1] and the major challenge of modern molecular neurochemistry is to identify and quantitate these substances in their various locations, to map their interconnections and to understand their modes of action. The trace amines (β-phenylethylamine, tryptamine, *m*- and *p*-tyramine, the octopamines, the synephrines and others) are a group of amine substances closely related to the bio-

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genic amines (dopamine, noradrenaline and serotonin) that are present in the nervous systems of all species studied to date. In vertebrates their concentrations are tiny, hence the neologism trace; in some invertebrates, however, they are present in much greater concentration and may in fact in some cases be one of the major neurotransmitters. The trace amines possess very fast turnover rates, are profoundly affected by psychoactive drugs and are synaptically and behaviourally active (see refs. 2–5 for further details and reviews). Our focus over the past few years has been to identify these substances in an unambiguous manner and then to develop quantitative procedures that are capable of routine use. Of necessity only limited aspects of the isolation, derivatization, separation, identification and quantitation of these amines and their principal metabolites can be considered here; those interested in investigating these substances further should read the quoted references to the original work.

SAMPLE PREPARATION

In neurochemistry there are many different ways in which a sample for analysis can be obtained depending on the type of analysis required. In straightforward whole brain or brain regional or peripheral nervous system analyses the animal is killed, the brain removed and various regions or nervous tissues are dissected out [6] although care must be taken with respect to the method of killing (cervical dislocation, microwave irradation, liquid nitrogen freezing, etc.) and the time taken to analysis. For those substances that cannot be mapped by conventional histofluorescent or immunological techniques because of their tiny concentrations, lack of reactive groups or because of immuno-crossreactivity with substances present in vast excess as is the case for some of the trace amines, a more detailed neuroanatomical analysis is possible by utilising finer dissections [7] or by slicing the brain followed by punching out nuclei of interest [8,9]. In these latter cases of course the amount of material available for analysis is very small. In studies on human brain, whilst biopsy samples can sometimes be obtained it is more usual to study brain regions obtained at autopsy or from a brain bank in which case it is necessary to be cognizant of the problems arising from age, sex, agonal state, time after death, preterminal medication, etc. [10]. Since neurotransmitter substances tend to be concentrated in the nerve endings (synaptosomes) whilst other substances such as enzymes, lipids, etc. are located in other cellular organelles or membranes, it is possible after homogenising the brain, or parts of it, in an isotonic medium, to fractionate the homogenate on a sucrose, Ficoll or Percoll gradient and isolate the cellular constituents (nuclei, mitochondria, synaptosomes, myelin, etc.) [11,12] for subsequent analysis. Further treatments (hypotonic disruption for example) will yield membrane and granular fractions. Finally of course nervous tissue can also be obtained by tissue culture techniques [13]. In more functional studies such as those aimed at establishing whether or not a substance is released after various electrical or chemical stimulations, it is possible to use slices or to obtain in vivo samples by inserting various collecting devices such as push–pull cannulae, superfusion caps or dialysis membranes [14] into specific locations in the brain.

ISOLATION

The overall procedures for the isolation, derivatisation, separation and qualitative and quantitative analyses of trace amines and their acidic metabolites are shown in Figs. 1 and 2, respectively. In the case of amines, depending upon the size of the sample, it can either be derivatised directly (≥ 100 mg or 0.5 ml) or after isolation of the amine fraction by ion-exchange or adsorption chromatography or after extraction at an alkaline pH value. The details of these procedures for the trace and other amines have been described in detail in several publications [2,7,15–31].

Acidic and neutral metabolites are extracted with ethyl acetate from depro-

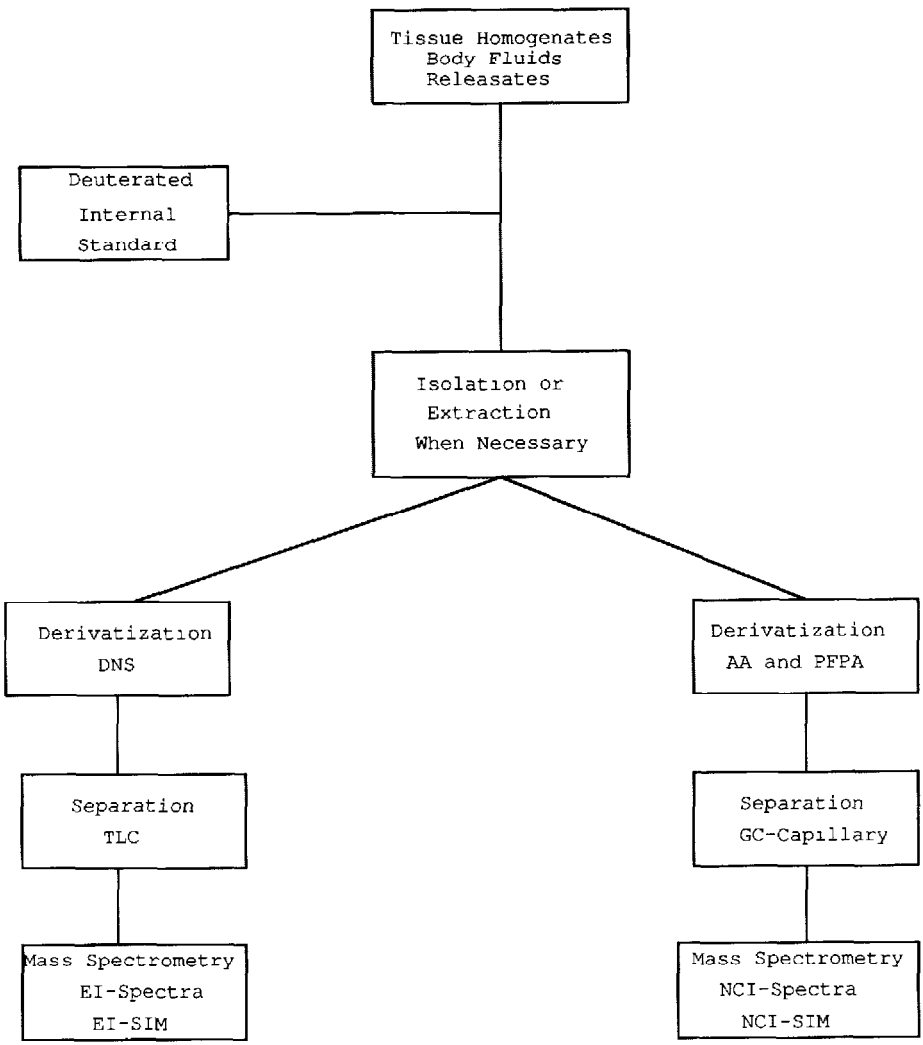

Fig. 1. Schematic flow chart for the qualitative and quantitative analysis of trace amines.

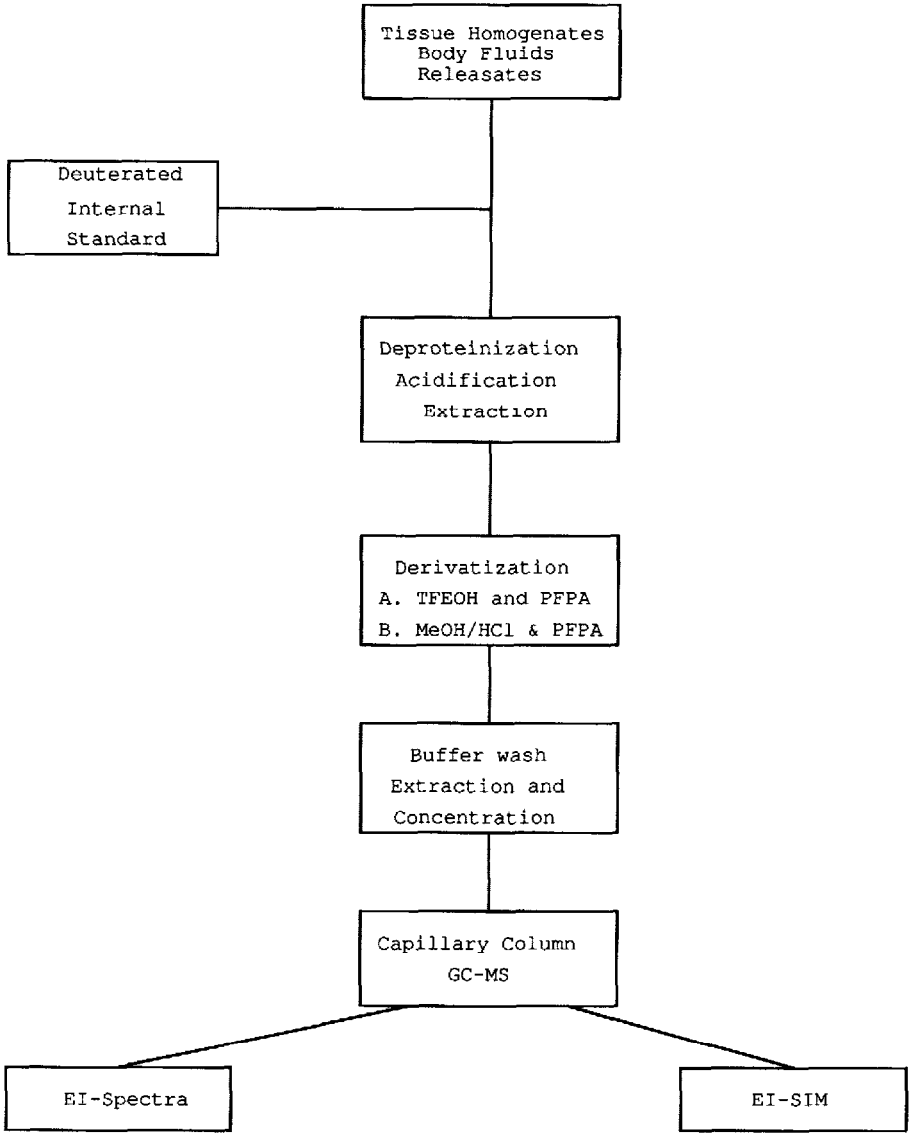

Fig. 2. Schematic flow chart for the qualitative and quantitative analysis of some acidic metabolites of biogenic amines.

teinised homogenates or fluids after acidification (see refs. 29–34 for further details).

Amines and acids in the above isolates, after derivatisation, are separated either by thin-layer chromatography (TLC) or gas chromatography (GC), and then identified by mass spectrometry (MS), at both low and high resolutions. Their concentrations can also be determined by comparing in the mass spectrometer the signal, usually of their molecular ion, with the signal produced by the molecular ion of an appropriate internal standard. In our work the internal standard is

always the amine or acid to be analysed suitably labelled with deuterium (see Tables III and IV). For this quantitative procedure to function properly the internal standard or mixture of them must be added to the homogenate or fluid to be analysed before any processing of the sample starts as shown in Figs. 1 and 2. Blanks usually of distilled water of a volume similar to the sample to be analysed and to which internal standards have been added are always processed in parallel as are varying amounts of the amines or acids added as supplements so as to yield an estimate of the recovery of the procedure. Since many biogenic amines and acids can exist in conjugated as well as unconjugated forms an estimate of the amount of the conjugated form can be obtained after hydrolysing (usually in strong acid or after enzyme hydrolysis) the sample. It is usual to analyse the sample for unconjugated amounts and then, after hydrolysis, for total amounts; subtraction of unconjugated from total yields the conjugated amount.

DERIVATISATION

Amines

When analysis is to involve MS, it is desirable that the chosen derivative possess certain characteristics; for example it should exhibit preferably an intense molecular ion or failing that, unique fragment ions. It is also an advantage when working with complex mixtures if the derivatives are relatively easily separable from each other so that some preliminary clean-up of the sample can occur before the derivatised amine to be analysed is introduced into the mass spectrometer (see Fig. 3 for an example). Many derivatives [trimethylsilyl (TMS), trifluoroacetyl, pentafluoropropionyl (PFP), heptafluorobutyryl, isothiocynate, pentafluorobenzylimine, flophemsyl, etc.] have been assessed but they all suffer from some deficiencies. The one we have adopted is the so-called dansyl (DNS, dimethylaminonaphthalenesulphonyl) derivative. Although the bansyl (N,N-dibutylaminonaphthalenesulphonyl) and propansyl (dipentylaminonaphthalenesulphonyl) derivatives are somewhat better with respect to their MS properties [35] (see Table I), the DNS reagent possesses the considerable merit of being commercially available and thus convenient to obtain and use. The dansylation procedure for small samples has been described in refs. 16–20 and 23–30. In brief the amine or mixture of amines to be analysed, in the presence of appropriate added deuterated internal standards in an alkaline medium, is mixed with an acetone solution of DNS reagent, left overnight at room temperature and then after concentration and extraction, the mixtures of derivatised amines are transferred to the origin zone of a silica gel plate and separated in the presence of appropriate DNS-amine standards run in parallel in one or more solvent systems (see Fig. 3 and Table II for details).

In situations where greater sensitivity (i.e., $> 10^{-12}$ g) is required than that offered by selected-ion monitoring (SIM) of DNS derivatives in the MS electron impact (EI) mode, it is possible at least for tryptamine, phenylethylamine and the tyramines to utilize negative chemical ionisation (NCI) GC–MS procedures. In a recently developed method [36] a perfluorinated spirocyclic derivative of the PFP derivative of N-acetyltryptamine (1-pentafluoro-2-methylenepyrolidine-3-

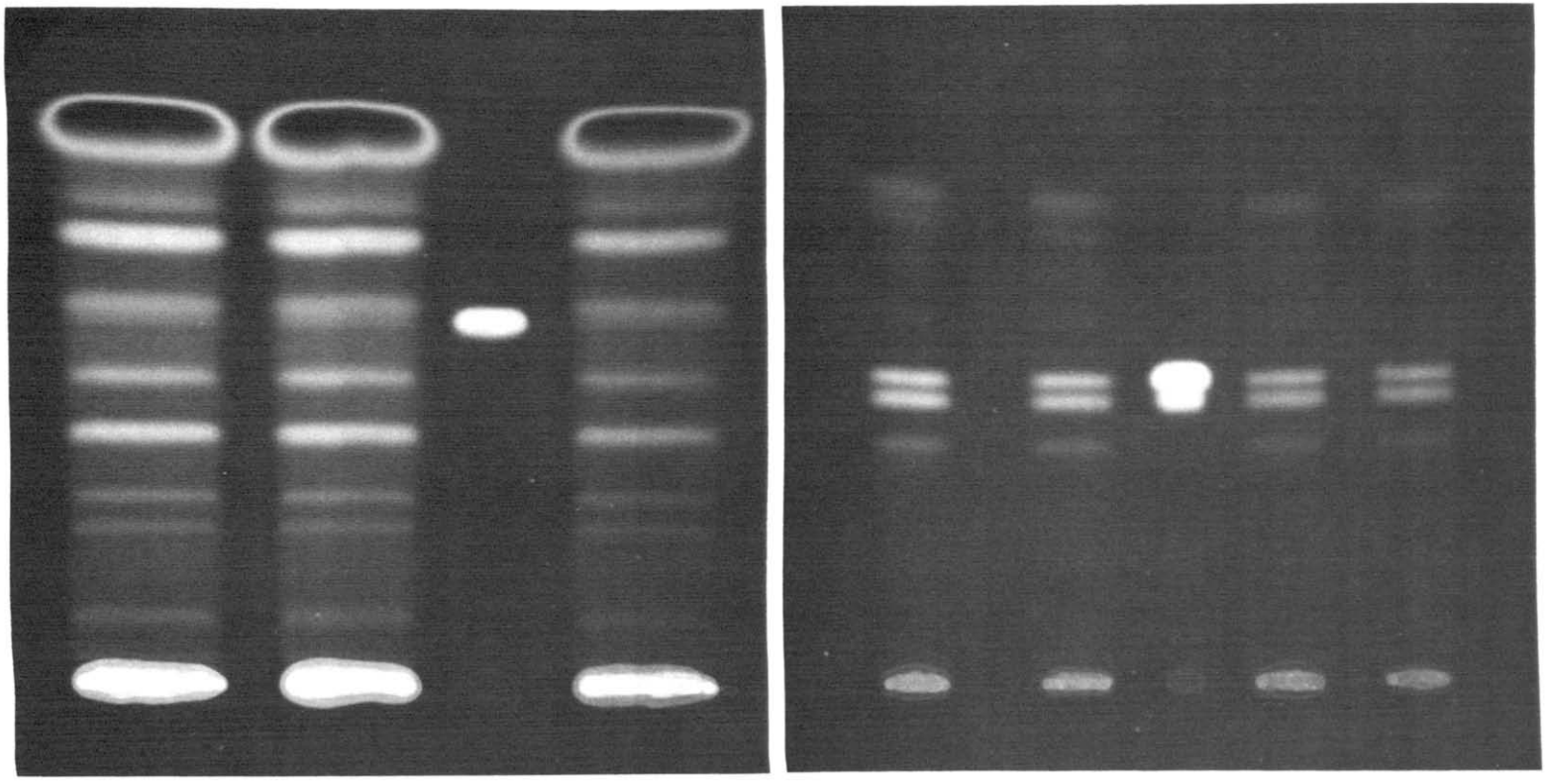

Fig. 3. Separation of DNS-*m*-tyramine and *p*-tyramine. On the left are several DNS-amines as present in an extract of pooled caudate nuclei from the rat. Standards of DNS-*p*-tyramine and DNS-*m*-tyramine are run on both plates as shown. In the second separation (right) it is clear that not only has the sample been considerably 'cleaned-up' but the *m*- and *p*-isomers of tyramine have also been resolved.

TABLE I

RELATIVE INTENSITIES AND MASSES OF THE MOLECULAR ION AND OTHER IONS OF CERTAIN N,N-DIALKYLAMINONAPHTHALENESULPHONYL DERIVATIVES OF *p*-TYRAMINE

These derivatives exhibit strong fluorescence in ultraviolet light and posses excellent chromatographic properties as illustrated in Fig. 3. The three shown in this table also exhibit ions in EI-MS that are suitable for quantitation by the MS-EI-IIC procedure as shown in Figs. 6 and 7. In this table the molecular ion and those arising by cleavage of the S–O bond and the $S-N_{naphthalene}$ bond (masses in brackets) are shown. Whilst, as can be seen, the bis-bansyl and bis-propansyl derivatives possess more intense molecular ions than does the bis-dansyl, the difference in practical terms is not that significant (see Table V) and in addition the bansyl and propansyl reagents are difficult to synthesize and thus use on a routine basis.

Derivative	Molecular ion (%)	S–O (%)	$S-N_{naphthalene}$ (%)
Bis-dansyl	7 (603)	6 (370)	100 (170)
Bis-bansyl	17 (771)	16 (454)	27 (254)
Bis-propansyl	19 (715)	19 (426)	18 (226)

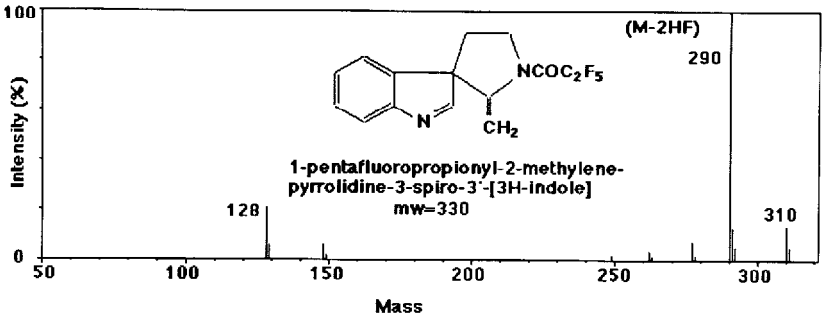

Fig. 4. NCI spectrum of the spirocyclic tryptamine derivative [1-pentafluoropropionyl-2-methylene-pyrolidine-3-spiro-3'-(3H-indole)] obtained on a 70-70F mass spectrometer with methane reagent gas. Shown is the structure of the derivative and the major M – 2HF ion which is used for quantitative purposes by SIM.

spiro-3'-3H-indole) can be detected at the 10^{-14} g level (see Fig. 4 for structure and mass spectrum). In this case very small samples containing tryptamine (i.e., from a single rat or mouse caudate nucleus or from punched out nuclei) are treated with acetic anhydride and pentafluoropropionic anhydride (PFPA) to yield PFP-N-acetyltryptamine which, when injected onto the gas chromatograph, cyclizes and elutes as the strongly electron-capturing spirocyclic tryptamine derivative shown in Figs. 4 and 5. Similar sensitivities can be obtained for phenylethylamine and the tyramines by using N-acetylpentafluorobenzoyl derivatives [37].

Acids

The acidic metabolites of biogenic amines, up to twelve of them, as they exist in tissue extracts or body fluids are, in our most recently developed procedure [34] converted in the case of phenylacetic acid (PAA), *m*-hydroxyphenylacetic (mPHA), *p*-hydroxyphenylacetic acid (pHPA) and homovanillic acid (HVA) to

TABLE II

SOLVENT SYSTEMS AND TLC R_F VALUES FOR SOME TRACE AMINES SEPARATED AS THEIR DNS DERIVATIVES

Solvents mixed on a volume for volume basis.

Amine	Solvent system 1	R_F value	Solvent system 2	R_F value
Phenylethylamine	Toluene–ethyl acetate (5:2)	0.67	Toluene–triethylamine (5:1)	0.53
Tryptamine	Toluene–ethyl acetate (5:2)	0.49	Toluene–triethylamine–methanol (50:5:7)	0.38
m-Tyramine	Toluene–ethyl acetate (5:2)	0.55	Toluene–triethylamine–methanol (50:5:1)	0.42
p-Tyramine	Toluene–ethyl acetate (5:2)	0.55	Toluene–triethylamine–methanol (50:5:1)	0.38
m-Octopamine	Chloroform–butyl acetate (5:3)	0.20	Benzene–triethylamine (5:2)	0.18
p-Octopamine	Chloroform–butyl acetate (5:3)	0.22	Benzene–triethylamine (5:2)	0.22
m-Synephrine	Chloroform–butyl acetate (5:3)	0.35	Benzene–triethylamine (5:2)	0.44
p-Synephrine	Chloroform–butyl acetate (5:3)	0.39	Benzene–triethylamine (5:2)	0.48

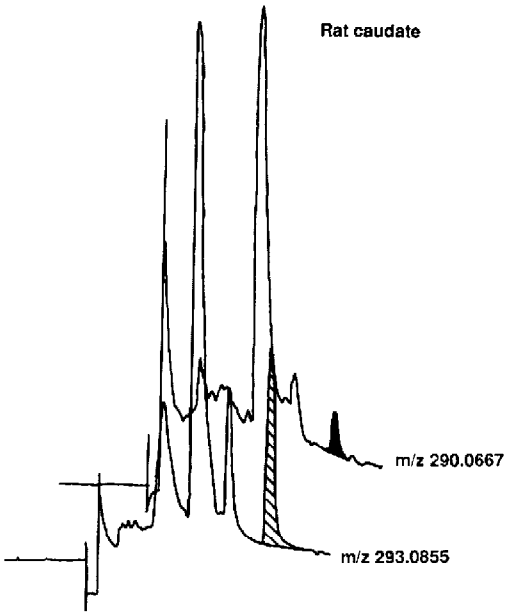

Fig. 5. GC–MS–NCI–SIM separation and analysis of the spirocyclic N-acetyl-PFP derivative of tryptamine and tryptamine-d_4 from a single caudate nucleus of the rat. The upper trace represents the SIM profile at the precise mass of the M − 2HF ion of the spirocyclic tryptamine derivative and the lower trace that of the derivative of $\alpha,\alpha,\beta,\beta$-tetradeuterotryptamine added as internal standard. Note that in this latter case one of the deuteriums is lost. See ref. 36 for further details.

their respective trifluoroethyl (TFE) ester-PFP derivatives or in the case of mandelic acid (MA), *m*-hydroxymandelic acid (mHMA), *p*-hydroxymandelic acid (pHMA), indoleacetic acid (IAA), dihydroxyphenylacetic acid (DOPAC), vanillylmandelic acid (VMA) and 5-hydroxyindoleacetic acid (5-HIAA) to their methyl ester-PFP derivatives followed by GC–MS–SIM. Briefly unconjugated or hydrolysed samples containing appropriate deuterated internal standards and about 10 μg each of phenylpropionic, *p*-hydroxyphenylpropionic and 5-methoxyindole-3-acetic acid added as carriers along with ascorbic acid and EDTA as antioxidants are deproteinized with sulfosalicylic acid and centrifuged. After extraction and transfer with ethyl acetate the samples are divided in half (aliquots A and B); each half is then concentrated to about 200 μl, transferred to Reactivials (Pierce, Rockford, IL, U.S.A.) and dried carefully and azeotropically in the presence of triethylamine and benzene. Aliquot A is then derivatized with methanolic HCl, dried carefully and the residue reacted with PFPA. Aliquot B is derivatised with the TFE and PFPA reagents and similarly dried carefully. The derivatised A and B aliquots are then recombined using hexane as solvent, washed with phosphate buffer and aliquots of the hexane solution injected onto a GC–MS capillary column. All details of the derivatisation, separation and MS analytical procedures will be found in ref. 34.

SEPARATION

DNS-amines are separated usually twice (see Fig. 3) on thin layers of silica gel in the solvent systems listed in Table II. After the final separation the amine to

TABLE III

GC–MS PARAMETERS USED IN THE ANALYSIS OF UP TO TWELVE BIOGENIC AMINE METABOLITES

Program	Reference mass	Metabolite derivative	Mass	Ion measured (elemental composition)	Retention time (min:s)
I	213.9903	PAA-TFE	H 218.0555	M^+	6:00
			d_5 223.0868	($C_{10}H_9F_3O_2$)	
II	213.9903	MA-Me-PFP	H 253.0288	$(M-COOCH_3)^+$	8:26
			d_5 258.0602	($C_{10}H_6F_5O_2$)	
III	375.9807	mHPA-TFE-PFP	H 380.0297	M^+	9:15
			d_4 384.0564	($C_{13}H_8F_8O_4$)	
		pHPA-TFE-PFP	H 380.0297	M^+	9:42
			d_4 384.0546	($C_{13}H_8F_8O_4$)	
		mHMA-Me-diPFP	H 415.0028	$(M-COOCH_3)^+$	10:34
			d_3 418.0216	($C_{13}H_5F_{10}O_4$)	
IV	375.9807	pHMA-Me-diPFP	H 415.0028	$(M-COOCH_3)^+$	11:40
			d_4 419.0279	($C_{13}H_5F_{10}O_4$)	
		DOPAC-Me-diPFP	H 415.0028	$(M-COOCH_3)^+$	12:08
			d_5 420.0342	($C_{13}H_5F_{10}O_4$)	
		MHPG-tri-PFP	H 445.0134	$(M-CH_2OCOC_2F_5)^+$	12 54
			d_1 446.0197	($C_{14}H_7F_{10}O_5$)	
		HVA-TFE-PFP	H 410.0400	M^+	13:02
			d_3 413.0589	($C_{14}H_{10}F_8O_5$)	
V	375.9807	VMA-Me-diPFP	H 445.0134	$(M-COOCH_3)^+$	14:10
			d_3 448.0322	($C_{14}H_7F_{10}O_5$)	
VI	325.9839	IAA-Me-PFP	H 335.0581	M^+	17:24
			d_2 337.0707	($C_{14}H_{10}F_5NO_3$)	
VII	375.9807	5-HIAA-Me-diPFP	H 438.0188	$(M-COOCH_3)^+$	18:39
			d_2 440.0314	($C_{15}H_6F_{10}NO_3$)	

be analysed, located by standards run in parallel, is eluted from the plate and identified and quantitated by MS as described below.

In the case of NCI analysis an aliquot of the spirocyclic tryptamine-acetyl-PFP derivative in hexane is injected onto a thin-film capillary column (J&W DBI, 60 m×0.32 mm I.D., 0.25-μm bonded phase) operated with a helium flow-rate of 33–40 cm/s and a temperature program of 170°C isothermal for 2 min, 10°C/min to 290°C and then isothermal for 4 min (see ref. 36 for complete details) (see Fig. 5).

For trace and other acids an aliquot of the recombined TFE-PFP and methyl-PFP derivatives is injected onto a GC (HP-5700)–MS capillary column (J&W bonded phase silica, DB-1, Chromatographic Specialties, Brockville, Canada, 60 m×0.32 mm I.D., 1-μm bonded phase column) with a helium flow-rate of 30 cm/s and operated isothermally at 140°C for 8 min and then increased by 10°C/min to 290°C and held for 8 min. The retention times and mass spectra of the acids along with their deuterated internal standards are shown in Table III and Fig. 6.

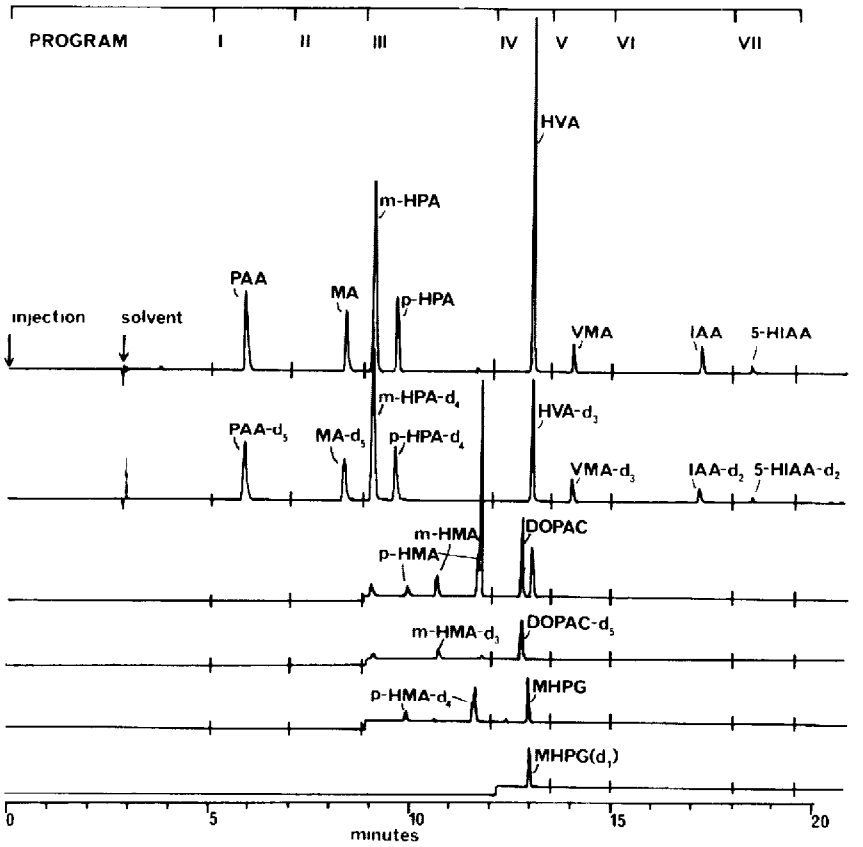

Fig. 6. Mass chromatogram of a mixture of biogenic amine metabolites and their deuterated internal standards. The derivatives used, their MS parameters and their GC retention times are shown in Table II. The VG 70-70F is regulated by the DIGMID multiple-ion detection controller and a magnet controller to select the reference mass within the mass spectrometer. See ref. 34 for further details.

MASS SPECTROMETRY

Direct probe electron impact

A high-resolution mass spectrometer (AEI MS902S, AEI, Manchester, U.K.) operated in the EI mode is used for the analysis of amines. An aliquot of the eluted DNS-amine derivatives is placed into the tip of a direct insertion probe and then introduced into the ion source region of the mass spectrometer. For identification purposes the mass spectrometer is operated at low resolution to produce spectra as shown in Fig. 7A. For quantitative analysis the mass spectrometer is set up at higher resolutions using the parameters listed in Table IV so as to record the so-called integrated ion current (IIC) (see Fig. 7B) of the molecular ion of the DNS-amines to be analysed and the molecular ion of the appropriate internal standard. Since the amount of deuterated internal standard added to the sample at the beginning of the extraction, derivatization and separation procedure is known, the amount of unknown amine is calculated by multiplying the ratio of the areas

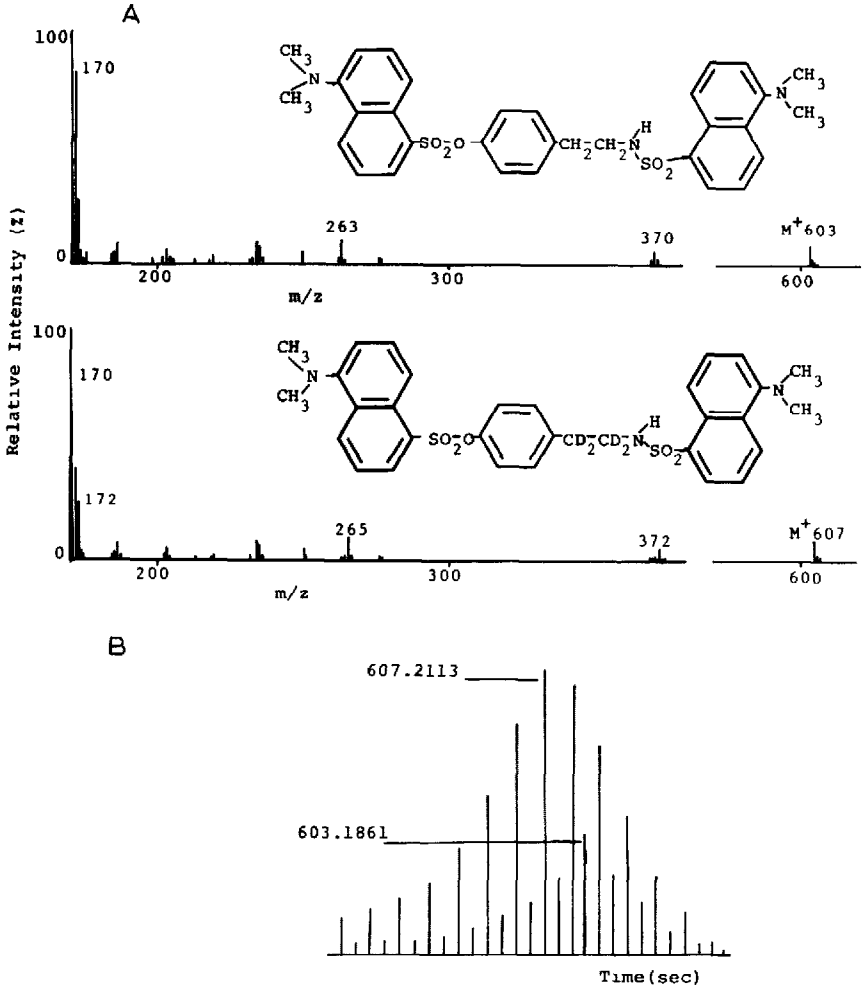

Fig. 7. (A) Mass spectra of the DNS derivatives *p*-tyramine and tetradeutero-*p*-tyramine (B) Integrated ion current (IIC) profile of the molecular ions of DNS *p*-tyramine and DNS *p*-tyramine-d_4.

of the two IIC curves by the amount of internal standard added. A similar procedure is used for the NCI analysis of tryptamine and for the various derivatives of the acidic metabolites. In this latter case, however, a double focusing VG 70-70F (VG Analytical, Manchester, U.K.) mass spectrometer is used along with the DIGMID program (VG Analytical) which correlates the appropriate MS reference mass with the appropriate molecular ion masses of the various derivatized acids and internal standards as they elute from the capillary column. The various achievable sensitivities and minimum detection levels for DNS-amines, spirocyclic derivatives of tryptamine and the methyl-PFP and TFE-PFP derivatives of acids in standard solution, tissue extracts and body fluids using MS techniques are shown in Table V. Complete details on the MS operating conditions and the various isotopic contributions will be found in refs. 29–34, 36 and 37.

TABLE IV

MS PARAMETERS USED IN THE QUANTITATION OF TRACE AMINES AS THEIR DNS DERIVATIVES

Amine	Reference mass	Mass	Elemental composition	Evaporation temperature (°C)
Phenylethylamine	363.9807	H 354.1402	$C_{20}H_{22}N_2O_2S$	250
		d_4 358.1653	$C_{20}H_{18}N_2O_2SD_4$	
Tryptamine	413.9775	H 393.1511	$C_{22}H_{23}N_3D_2S$	260
		d_4 397.1762	$C_{22}H_{19}N_3O_2SD_4$	
m-Tyramine	613.9647	H 603.1861	$C_{32}H_{33}N_3D_5S_2$	280
		d_4 607.2113	$C_{32}H_{29}N_3O_5S_2D_4$	
p-Tyramine	613.9647	H 603.1861	$C_{32}H_{33}N_3D_5S_2$	280
		d_4 607.2113	$C_{32}H_{29}N_3O_5S_2D_4$	

TABLE V

MINIMUM DETECTION LEVELS OF SOME TRACE AMINES AND ACIDS

Substance	Derivative	Minimum detectable amount[a]	
		From standard solutions (pg)	From tissue extracts (pg)
TLC-EI-SIM procedure			
Phenylethylamine	Dansyl	2–5	100
Tryptamine	Dansyl	2–5	100
Tyramines	Dansyl	2–5	100
NCI-SIM procedure			
Tryptamine	Spirocyclic N-acetyl-PFP	0.16	1–10
GC-EI-SIM procedure			
PAA	TFE	15	300
MA	Me-PFP	5	100
mHPA	TFE-PFP	10	150
pHPA	TFE-PFP	10	200
mHMA	Me-diPFP	–	N.D.[b]
pHMA	Me-diPFP	5	100
VMA	Me-diPFP	15	350
HVA	TFE-PFP	10	200
DOPAC	Me-diPFP	25	1000
MHPG	tri-PFP	25	1000
IAA	Me-PFP	5	500
5-HIAA	Me-diPFP	50	2000

[a]Amount on probe or injected.
[b]N.D. = not detected.

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