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THE ANALYSIS OF CERTAIN AMINES IN TISSUES AND BODY FLUIDS AS THEIR DANSYL DERIVATIVES

ALAN A. BOULTON, S. R. PHILIPS and D. A. DURDEN
Psychiatric Research Unit, University Hospital, Saskatoon, Saskatchewan (Canada)

SUMMARY

A general procedure for the isolation and chromatographic separation of some biogenic amines, as their Dansyl* derivatives, and their quantitative evaluation by a mass spectrometric procedure is described. In the case of β-phenylethylamine, which has been used as a typical example, the endogenous tissue concentrations (ng/g) in the rat were: spleen, 4.7 ± 2.7; lung, 4.0 ± 1.4; liver, 2.0 ± 0.7; kidney, 20.5 ± 2.2; heart, 5.7 ± 3.1; and brain 1.8 ± 0.4. After treatment with various monoamine oxidase inhibitors these values increased between 7 and 198 times.

INTRODUCTION

Because of the availability of suitable sensitive fluorimetric procedures the catecholamines noradrenaline and dopamine, and the indoleamine 5-hydroxytryptamine, along with some of their metabolites, have been identified and quantitated in the tissues and body fluids of most species. As a consequence, much is known about their excretion in 'normal' individuals, in individuals stressed in a variety of ways and in many pathological conditions. Similarly, the endogenous concentration in numerous tissues and the effect of many drugs on these levels have been investigated. The anatomical and subcellular distribution patterns were established following the development of histofluorescent and autoradiographic techniques.

More recently attention has turned to other amines. Urinary unconjugated *p*-tyramine, for instance, is known to be excreted in abnormal amounts by patients suffering from Parkinson's disease[1–3] and schizophrenia[4]. Furthermore, the urinary levels appear to correlate with cerebral electrical activity as measured from surface electrodes[5]. *p*-Tyramine has been identified in rat brain[6] and is synthesised intracerebrally by dehydroxylation of dopamine and presumably decarboxylation of tyrosine[7–9]. Endogenous levels of β-phenylethylamine have only very recently been quantitated[10,11]. This amine has, however, been identified in a variety of mammalian tissues after pretreatment with monoamine oxidase inhibitors and/or phenylalanine[12,13]. It has been shown that this amine is excreted in the urine in increased quantities by patients suffering with phenylketonuria[14] and perhaps in decreased amounts by patients suffering with depressive illnesses[15–17].

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* Dansyl = 5-dimethylaminonaphthalene-1-sulphonyl chloride.

As long ago as 1962 Brune and Himwich[18] claimed that variations in the urinary excretion of tryptamine correlated with exacerbations of aberrant behaviour in schizophrenia. More recently, it has been claimed that certain methylated derivatives of tryptamine are excreted uniquely by schizophrenics[19,20]. Enzymes capable of methylating indoleamines have certainly been identified in some mammalian tissues[21–26]. Some values for endogenous tissue tryptamine, both in the presence and absence of drugs, have been reported[27–30].

In this laboratory we have been interested in the above-mentioned amines for some time and have attempted to analyse them as their respective Dansyl derivatives. Although much chromatographic information has been obtained, it is difficult to be sure of a complete separation from other metabolites and contaminants, and hence of quantitation in absolute terms. Separation of the Dansyl derivatives in two or more solvent systems, however, followed by certain mass spectrometric measurements, such as the mass spectrum and precise mass analysis, increases this certainty. In this way β-phenylethylamine, tryptamine and tyramine have been shown to be present in all tissues of the rat analysed so far and the endogenous distribution of phenylethylamine has been established. For the purposes of this review, the analytical procedure is briefly outlined using phenylethylamine as an example. Further data will be published elsewhere.

MATERIALS AND METHODS

All chemicals and solvents were of the purest grade commercially available. We thank Dr. N. Seiler (Frankfurt, G.F.R.) for crystalline Dansyl-phenylethylamine, Dr. Bruce Davis (Saskatoon, Canada) for the synthesis of 1,1-dideutero-2-phenylethylamine, Abbot Laboratories (Chicago, Ill., U.S.A.) for pargyline and Lakeside Laboratories (Milwaukee, Wisc., U.S.A.) for catron. Iproniazid phosphate was purchased from the Sigma Chemical Co., St. Louis, Mo., U.S.A.

Male Wistar rats (body weight 150–200 g), some injected intraperitoneally with pargyline, catron or iproniazid (see Table III for dosages), were stunned and decapitated, and the liver, lungs, kidneys, spleen, heart and brain removed. After chilling, blotting and weighing, the tissues were homogenised at 0° in 20 ml 0.4 *N* $HClO_4$. Deuterated phenylethylamine (1,1-dideutero-2-phenylethylamine, 25 ng or 100 ng) was added as an internal standard. The suspension was mixed and centrifuged at 12,000 $\times g$ for 10 min. Triton X-100 (J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.) was added to the supernatant to a final concentration of 0.2% w/v in order to prevent association of phenylethylamine and tryptamine with particulate lipoprotein materials[31]. Following adjustment to pH 7.0, the solution was percolated through a column (1.5 cm diameter $\times$ 3 cm long) of Bio-Rad AG 50W-X2 (H^+ form). The resin was washed successively with water (10 ml), 0.1 *M* sodium acetate (20 ml) and water (10 ml), and the amine fraction eluted with 10 ml methanol–HCl (73:27) [10 ml ethanol–ammonia–water (65:25:10) in the case of tyramine and tryptamine], rotary evaporated to dryness and then dissolved in 1 ml of sodium carbonate (10% w/v). The Dansyl derivatives of the amines were prepared by mixing this solution with 1.5 ml of Dansyl reagent (5-dimethylamino-1-naphthalene sulphonyl chloride, Calbiochem, Los Angeles, Calif., U.S.A.; 1 mg/ml in acetone) and allowing the reaction to proceed for 2.5 h at 37°. Sodium carbonate was precipitated by adding 9 ml of acetone. The clear supernatant was removed, rotary evaporated to dryness at 45° and

quantitatively transferred in a few drops of benzene–acetic acid (99:1) to a 20 × 20 cm thin layer of silica gel on glass (Mondray Ltd., Montreal, Canada). Several tissue samples and standards of Dansyl-phenylethylamine were accommodated on the same plate. The chromatograms were developed in one of the solvent systems listed below. All chromatography tanks were of standard size (length 21 cm, width 8.5 cm and depth 20.5 cm) and were maintained at a temperature of 21 ± 1°. Solvents were freshly mixed just before use. When the solvent front was about 2–3 cm from the top of the layer, the plates (two per tank) were removed, and whilst still damp, sprayed with isopropanol–triethanolamine (4:1) in order to stabilise the fluorescence of the Dansyl-derivatives[32]. The appropriate zone, as assessed from standards run in parallel, was outlined in pencil under ultraviolet light (365 nm), scraped from the layer, eluted in 3 ml of benzene–acetic acid (99:1), rotary evaporated to dryness and transferred in benzene–acetic acid (99:1) to a second Mondray silica gel plate. After a second separation, the Dansyl-amine zone was similarly stabilised, eluted and transferred, but this time to a thin layer of silica gel on polythene (Kodak No. 6061). At the end of a third separation the appropriate zone was quickly scraped off and eluted, but in the absence of fluorescence stabilisation. The final extracts, containing Dansyl-phenylethylamine or Dansyl-tryptamine, were dried and redissolved in 500 μl of redistilled absolute ethanol containing 0.2% (v/v) acetic acid. Aliquots (5 μl) were subjected to mass spectrometric analysis[11]. Analysis of Dansyl-*p*-tyramine was somewhat modified; the zone from the final chromatogram was removed and deposited in a capillary tube as described by Seiler[33], eluted into 25 μl of ethyl acetate and a 5-μl aliquot subjected to mass spectrometry.

The solvent systems (see below) found to be the most suitable in separating the three amines of interest from all other Dansyl reactive metabolites and contaminants were, respectively: phenylethylamine, 1, 2, 3; tryptamine, 1, 4, 5; and tyramine, 1, 4 and 6. The final separation of each Dansyl-amine was always carried out on a Kodak plate because the fluorescence decay and oxidation of the derivatives in the absence of stabilisation, as well as the background contamination in the mass spectrometer, were least marked on this medium. Although the relative R_F values of the Dansyl-amines remain the same on the various commercially available silica gel coated thin media, the absolute values may change somewhat from layer to layer. The solvent systems utilized are listed in Table I.

Details of the mass spectrometric analysis have been published elsewhere[10,11]. Spectra were recorded on an AEI MS-902S mass spectrometer equipped with a direct insertion inlet and Massmaster mass indicator, and operated at 70 eV. The source temperature for the analysis of Dansyl-phenylethylamine was 260 ± 10°. Quantitative estimations were obtained using the integrated ion current procedure at a resolution of 10,000. The peak switching mechanism was arranged so to record the ion currents at m/e 356.1527 (molecule ion of Dansyl-1,1-dideutero-2-phenylethylamine), m/e 354.1402 (molecule ion of Dansyl-phenylethylamine) and m/e 363.9807 (reference ion of heptacosafluoro-tri-*n*-butylamine). The tissue level of phenylethylamine was calculated from the ratio between deuterated Dansyl-phenylethylamine added as internal standard (25 ng for endogenous calculations and 100 ng when drugged rats were used) and the Dansyl-phenylethylamine itself after subtracting the isotopic contribution of ^{13}C, ^{34}S and ^{18}O associated with the Dansyl-phenylethylamine. Further details of this procedure are listed in ref. 11.

RESULTS AND DISCUSSION

Some qualitative chromatographic data with respect to the three amines in question are listed in Table I. It must be remembered that the number of Dansyl derivatives formed on derivatisation of tissue extracts is large. Removal of the zones of interest followed by further separation certainly reduces the number of isographic fluorophores, but in some cases, even after three separations the final extracted fluorophore was contaminated. Dansyl-amines are visible after ultraviolet excitation at the 5-ng level. Furthermore, they exhibit a linear relationship between concentration and amount of fluorescence, at least in the 10^{-8}–10^{-5} g range. Care must be taken if direct fluorimetric or photodensitometric evaluation of the final separated fluorophore is attempted, however, because misleading values may be obtained.

TABLE I

R_F CHARACTERISTICS OF SOME DANSYL AMINE DERIVATIVES

Values listed for solvents 1, 2, and 4 were obtained using Mondray plates; those listed for solvents 3, 5, and 6 were obtained using Kodak plates. Other commercially available plates produced similar patterns but different absolute values.

No.	*Solvent system**	*Dansyl-Phenylethylamine*	*Dansyl-Tyramine*	*Dansyl-Tryptamine*
1	Chloroform–*n*-butyl acetate (5:2)	0.76	0.52	0.45
2	Benzene–triethylamine (8:1)	0.55	0.40	0.09
3	Light petroleum (b.p. 100–120°)–toluene–acetic acid–water (133:67:170:30)	0.38	0.20	0.12
4	Benzene–methanol (40:1)	0.64	0.56	0.36
5	Cyclohexane–ethyl acetate (5:2)	0.55	0.30	0.30
6	Cyclohexane–ethyl acetate (3:2)	0.69	0.56	0.50

* Solvents were freshly mixed on a volume-to-volume basis before use.

If quantitation is obtained instead by evaporating the derivative in the mass spectrometer focused at a precise mass, followed by assessment of the area of the integrated ion current curve, the specificity is considerably enhanced. Indeed, in this latter case, it is quite possible to obtain a specific analysis after only a single chromatographic separation, provided substantial suppression of the ionisation does not occur. Calculating the amount of amine (and this technique can be applied to any volatile substance) by using the ratio between it and the same amine suitably labelled with a stable isotope (deuterium in this case) obviates the need for calibration curves. Since the internal standard is added to every single specimen, all parameters affecting the accuracy of analysis, such as recovery from the ion-exchange column and/or the chromatograms, the extent of derivatisation and changes in the instrument are taken into account. Tissue levels of β-phenylethylamine in untreated rats and rats pre-treated with the monoamine oxidase inhibiting drugs pargyline, catron and iproniazid are listed in Tables II and III.

TABLE II

ENDOGENOUS LEVELS OF β-PHENYLETHYLAMINE IN SOME TISSUES OF THE RAT

Tissue	*β-Phenylethylamine (ng/g)**
Brain (n = 7)	1.8 ± 0.4
Heart (n = 9)	5.7 ± 3.1
Kidney (n = 8)	20.5 ± 2.2
Liver (n = 9)	2.0 ± 0.7
Lung (n = 9)	4.0 ± 1.4
Spleen (n = 9)	4.7 ± 2.7

* Mean ± standard deviation.

TABLE III

β-PHENYLETHYLAMINE LEVELS IN SOME TISSUES OF RATS PRE-TREATED WITH SOME MONOAMINE OXIDASE INHIBITORS

Drug	*Phenylethylamine (ng/g)* §					
	Brain	*Heart*	*Kidney*	*Liver*	*Lung*	*Spleen*
Pargyline* (n = 4)	136 ± 23	103 ± 9	782 ± 151	386 ± 48	297 ± 14	172 ± 33
Catron** (n = 5)	38 ± 9	39 ± 14	284 ± 56	84 ± 27	91 ± 18	72 ± 16
Iproniazid*** (n = 5)	112 ± 23	80 ± 10	804 ± 133	296 ± 44	439 ± 176	126 ± 25

* Pargyline dissolved in saline injected intraperitoneally (75 mg/kg) 4 h before sacrifice.
** Catron, in saline, injected i.p. (16 mg/kg) 4 h before sacrifice.
*** Iproniazid phosphate, in saline, injected i.p. (100 mg/kg) 4 h before sacrifice.
§ Mean ± mean deviation.

It can be seen that the endogenous levels of β-phenylethylamine are very low, but that after treatment considerable increases occur. These very large increases are suggestive of a very active synthesis and turnover rate for this particular amine. It is also of interest and somewhat surprising that the values listed in Table III, *i.e.* those obtained 4 h after injection of the drugs, should vary from drug to drug, since each of these drugs are supposed to be irreversible inhibitors of monoamine oxidase and a supposed excess was injected.

For the future we propose to continue the analysis of certain amines and their metabolites in tissues and body fluids obtained from a variety of species, including *post-mortem* human samples and samples obtained at biopsy. The effect of a variety of drugs useful in psychiatry and neurology on these amine levels will also be investigated. In order that the concentration of amines in very small tissue samples and/or subcellular fractions may be analysed, we propose to attempt to extract the chromatographically separated derivatives in volumes of solvent sufficiently small to be introduced directly into the mass spectrometer. Further increases in sensitivity are expected to accrue as a result of modifications to the chromatographic procedure such as a reduction in the dimensions of the thin layers and by the use of circular media[34,35].

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