Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 11/12, pp. 1145–1149, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

The determination of biogenic amines in four strains of the fruit fly *Drosophila melanogaster**

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Abstract: A range of biogenic amines were measured in the heads from four strains of *Drosophila melanogaster*. Quantitation was carried out using gas chromatography–negative ion chemical ionization mass spectrometry (GC–NICIMS) with stable isotope dilution. The principal amines detected in the heads were dopamine, noradrenaline and 5 HT with small amounts of *p*- and *m*-tyramine; *p*-octopamine could not be detected in samples of 25 heads with a limit of detection of 10 pg per sample. In addition to conventional neurotransmitters or putative neurotransmitters the amines 5- and 6-hydroxydopamine were detected in the heads in substantial amounts.

Keywords: *Gas chromatography–mass spectrometry;* Drosophila*; dopamine; noradrenaline; hydroxydopamines.*

Introduction

Drosophila melanogaster serves as a powerful model system in which to study the genetic basis of a variety of processes through the availability of a wide range of flies with single gene defects. Several mutant strains which have defects in pigmentation also have defects in genes involved in the biosynthesis of catecholamines [1]. Behavioural mutants may be deficient in key enzymes involved in biogenic amine biosynthesis and consequently might show variations in the concentrations of various biogenic amines in their neural tissues [2–4]. The neurotransmitters in the biogenic amine class that have been detected in *D. melanogaster* include *p*-octopamine, dopamine and 5 HT [1, 5, 6] with evidence for the presence of noradrenaline being rather contradictory [1, 5–8]. The presence of genes in *D. melanogaster* include coding for DOPA decarboxylase and tyrosine hydroxylase, which are key enzymes involved in the biosynthesis of biogenic amines, has been established [9, 10]. It has been proposed that cuticle tanning and neurotransmitter biosynthesis may be controlled separately via the action of tyrosinase and tyrosine hydroxylase (TH), respectively [1]; both enzymes carry out the orthohydroxylation of tyrosine to produce DOPA but tyrosinase is much less substrate specific. One might expect variations in the pigmentation of the insects to be linked to variations in tyrosinase activity rather than TH activity but experimental evidence does not entirely support this view since deficiency in TH causes a variation in pigmentation [1].

The predominant route of catabolism of biogenic amines in *D. melanogaster* is through the formation of *N*-acetates [1, 11, 12] as is the case in locust [13]. There is little evidence of COMT or MAO activity in the insects [1]. However, another route of metabolism of the amines exists, the formation of *N*-β-alanyl conjugates; the formation of these metabolites appears to be linked to the process of cuticle tanning [1].

In the current paper we report the analysis of biogenic amines in four strains of *D. melanogaster* by gas chromatography–negative ion chemical ionization mass spectrometry (GC–NICIMS).

Experimental

Chemicals

All solvents used were HPLC grade (Rathburn Chemicals, Peebleshire, UK). Chemicals were obtained from the following sources: 3, 4-dimethoxybenzaldehyde, 3-methoxybenzalde-

*Presented at the 'Fourth International Symposium on Pharmaceutical and Biomedical Analysis', April 1993, Baltimore, MD, USA.

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hyde, 4-methoxybenzaldehyde, dopamine, 6-hydroxydopamine, 5-hydroxydopamine, *p*-octopamine, *m*-octopamine and *p*-tyramine from Aldrich Chemical Co. (Dorset, UK); 3,5-ditrifluoromethylbenzyl chloride from DTFMBCl, Fluorochem (Derbyshire, UK); and *N,O*-bistrimethylsilylacetamide from BSA, Fluka (Derbyshire, UK). Other amines were either purchased or prepared as described previously [14].

Deuteriated internal standards

Most deuteriated internal standards were available from previous work [15–17]. [$^2H_5$] 5- and 6-hydroxydopamine and [$^2H_6$] dopamine were synthesized by standard methods [14] involving reaction of the appropriate methoxybenzaldehyde with [$^2H_3$] nitromethane with in $CH_3O^2H_1$ to yield a nitrostyrene, followed by reduction with $LiAl^2H_4$ to the amine and then cleavage of the methoxy ether groups with ^2H_1Br which resulted in simultaneous exchange of the aromatic protons for deuterium.

Biological materials

Three wild type strains of *D. melanogaster*: (Canton S; Novosibirsk and Sierra Leone) and one mutant strain lacking all visual tissue (*eya S1* [18]) were used in this study. The flies were cultured on standard cornmeal agar. They were then harvested, frozen in liquid N_2 and the heads were separated from bodies and appendages by sieving in liquid N_2, and stored at −20°C until analysis.

Sample extraction and derivatization

Twenty-five heads were counted out using a binocular microscope. The heads were transferred to a ground glass homogenizer, deuteriated internal standards were added [5 ng of each component (Table 1) in 5 μl of acetonitrile] and the sample was homogenized in 0.1 M HCl (0.5 ml). The sample was centrifuged, the supernatant removed and potassium phosphate buffer was added (0.5 ml, 1 M pH 7.4).

Ditrifluoromethylbenzoyl chloride (3 μl) was added and the sample was shaken for 5 min on a wrist action shaker. The sample was then extracted with ethyl acetate (2 ml) and the organic layer was removed; ammonia solution (0.5 ml, 10 M) was added and the sample was again shaken for 5 min. The organic layer was removed and dried by passing through anhydrous sodium sulphate (*ca* 3 cm in a Pasteur pipette). The sample was transferred to a reactivial, the ethyl acetate was then removed under a stream of nitrogen, BSA (30 μl) was added and the sample was heated at 60°C for 15 min. Finally, ethyl acetate (50 μl) was added and an aliquot of this sample (4 μl) was injected into the GC–MS.

Instrumental conditions

GC–MS analysis was carried out in the NICI mode using a Hewlett–Packard 5988 GC–MS system using the conditions described previously [15]. The GC was fitted with a 30 m × 0.25 mm i.d. × 0.22 μm film thickness Restek Rtx-1 capillary column (Belmont Instruments, Glasgow). The GC oven was held at 100°C for 1 min and then programmed at 10° min^{-1} to 300°C.

Results and Discussion

This is the first application of GC–NICIMS

Table 1
Deuteriated and undeuteriated standards used in quantitation of amines in *Drosophila melanogaster* heads with the ions used for quantification

Biogenic amine	(*m/z*)	Deuteriated int. std	(*m/z*)
m-Tyramine	449	[$^2H_6$] *m*-Tyramine	455
p-Tyramine	449	[$^2H_6$] *p*-Tyramine	455
m-Octopamine	537	[$^2H_3$] *m*-Octopamine	540
p-Octopamine	537	[$^2H_3$] *p*-Octopamine	541
Dopamine	537	[$^2H_6$] Dopamine	543
m-Synephrine	551	[$^2H_3$] *m*-Synephrine	554
p-Synephrine	551	[$^2H_3$] *p*-Synephrine	554
Normetanephrine	567	[$^2H_3$] Normetanephrine	570
Metanephrine	581	[$^2H_3$] Normetanephrine	570
Nordrenaline	625	[$^2H_3$] Noradrenaline	628
6-Hydroxydopamine	625	[$^2H_4$] 6-Hydroxydopamine	629
5-Hydroxydopamine	625	[$^2H_4$] 5-Hydroxydopamine	629
Adrenaline	639	[$^2H_3$] Adrenaline	642
5 HT	560	[$^2H_4$] 5 HT	564

Table 2
Average concentrations of biogenic amines extracted from the heads of four strains of *Drosophila melanogaster* (expressed as ng/25 heads ± SD)

	eya S1	Canton S	Novosibirsk	Sierra Leone
m-Tyramine	0.025 ± 0.005 (n = 6)	0.016 ± 0.001 (n = 4)	(—)	0.024 ± 0.004 (n = 6)
p-Tyramine	0.049 ± 0.007 (n = 5)	0.041 ± 0.019 (n = 5)	0.054 ± 0.008 (n = 5)	0.215 ± 0.135 (n = 2)
P-Octopamine	(—)	0.013 (n = 1)	0.026 (n = 1)	(—)
Dopamine	5.513 ± 0.729 (n = 6)	3.420 ± 0.923 (n = 7)	5.158 ± 0.826 (n = 6)	6.377 ± 1.794 (n = 6)
Noradrenaline	0.447 ± 0.109 (n = 6)	0.310 ± 0.050 (n = 7)	0.709 ± 0.145 (n = 6)	0.686 ± 0.222 (n = 6)
6-Hydroxydopamine	0.235 ± 0.052 (n = 4)	0.159 (n = 1)	0.901 (n = 1)	0.264 ± 0.112 (n = 6)
5-Hydroxydopamine	0.988 ± 0.764 (n = 6)	0.540 ± 0.220 (n = 7)	0.961 ± 0.287 (n = 6)	1.286 ± 0.370 (n = 6)
5-HT	0.179 ± 0.058 (n = 6)	0.220 ± 0.024 (n = 7)	0.235 ± 0.051 (n = 6)	0.241 ± 0.073 (n = 6)

n = Number of samples run, each containing 25 heads, in which the amine was above the limit of detection.
(—) = not detected; limits of detection in the range 5–15 pg per sample.

to the analysis of biogenic amines in nervous tissue from *Drosophila*. The ions monitored for the different amines and their deuteriated internal standards are shown in Table 1. The analytical procedure used for the analysis of these amines by GC–MS is well established [15, 19]. Table 2 summarizes the concentrations of biogenic amines found in samples of 25 heads of the four strains of *D. melanogaster*. Figure 1(A) shows the large peak for dopamine that was present in the extract from *D. melanogaster* heads. Figure 1(B) shows a region of Fig. 1(A) expanded over a narrower time range, thus enabling the expansion of the *Y*-axis by omitting the large dopamine peak. The trace indicates a very small peak for *p*-octopamine which, in this case, was no higher than the background (5 pg); *p*-octopamine was generally absent from the heads (limit of detection *ca* 10 pg per sample). Some of the other peaks in Fig. 1(B) may correspond to dihydroxyphenylethylamines (dopamine isomers). We have previously observed that a number of these are present in human urine [14]. The absence of *p*-octopamine is surprising since we have previously found considerable amounts of this neurotransmitter in cockroach brain and the thoracic ganglion of the locust [15, 19]. However, it appeared to be present less consistently in locust brain (unpublished observations). The localization of *p*-octopamine in specific neurons may mean that it is not evenly distributed throughout the insect's body.

Small amounts of *m*- and *p*-tyramine were found to be present in the fruit fly heads, we have previously found large amounts of *p*-tyramine both in cockroach brain [15] and in the thoracic ganglion from the locust [19]. The very low amounts of *p*-tyramine, which may be a precursor of *p*-octopamine, may correlate with the absence of *p*-octopamine from the heads.

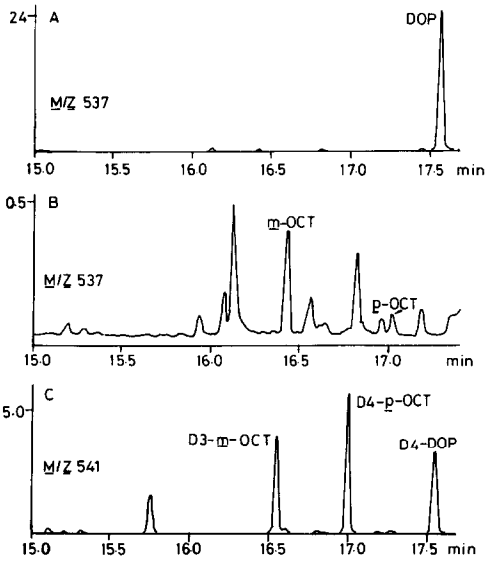

Figure 1
(A) SIM trace showing dopamine (*m/z* 537) extracted from 25 heads of *D. melanogaster* and converted to a DTFMB–TMS derivative. (B) Expanded portion of *m/z* 537 trace enabling *Y*-axis expansion to show the very small amounts of *p*-octopamine present. (C) SIM trace (*m/z* 541) showing [$^2H_4$] amines; a mixture of ^2H species are present for a given amine with the labelled *p*-octopamine being predominantly [$^2H_4$]; 5 ng of each ^2H amine was added to the *D. melanogaster* heads prior to extraction.

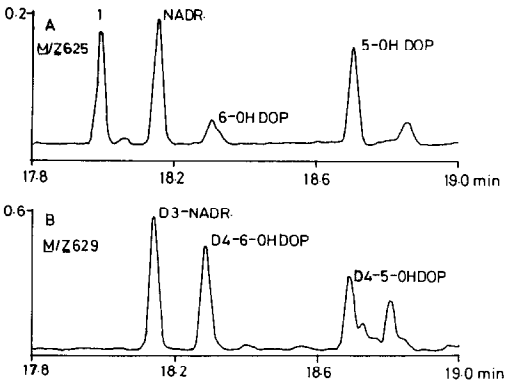

Figure 2
(A) SIM trace (*m/z* 625) showing peaks for noradrenaline and 5- and 6-hydroxydopamines extracted from 25 heads of *D. melanogaster* and converted to DTFMB–TMS derivatives. (B) SIM trace (*m/z* 629) showing ions derived from [$^2H_3$] noradrenaline and [$^2H_4$] hydroxydopamines; 5 ng of each ^2H amine was added to the *D. melanogaster* heads prior to extraction.

Figure 2(A) shows the SIM traces obtained when the ion for noradrenaline, and its isomers the hydroxydopamines, is monitored. Noradrenaline was readily detected in the fruit fly heads. Thus the main neurotransmitters in the heads, which are substantially composed of brain tissue, are dopamine and noradrenaline which corresponds to the findings in mammalian brains, e.g. rat (unpublished observations). 5-Hydroxydopamine and to a lesser extent 6-hydroxydopamine were detected in the heads; 2-hydroxydopamine may also be present in some instances. It has been reported previously that unidentified catecholamines are present in *D. melanogaster* and that fluctuations in the levels of these catecholamines are linked to different developmental stages of the insect [1]. This in turn may relate to the involvement of catecholamines in cuticle development. In addition to 5- and 6-hydroxydopamine, another isomer [peak 1, Fig. 2(A)] of these compounds and noradrenaline is consistently present in the insect tissues. We have also observed this compound in human urine [14].

We did not detect *m*-octopamine, *m*- and *p*-synephrine, normetanephrine, metanephrine or adrenaline in the fruit fly heads. Limits of detection were in the region of 5–15 pg per sample. The concentrations of noradrenaline and dopamine were in the region of those we have determined previously in nervous tissue from other insects [15, 19]. The concentrations of *p*-tyramine were much lower than those previously measured [15, 19]. The fact that we detected noradrenaline in the extracts whereas previous workers did not consistently detect it is probably due to the higher degree of specificity of our method; previous work has most frequently been based on non-specific radio enzyme assays combined with TLC separation. Our failure to detect *p*-octopamine can be similarly explained.

5-HT was consistently detected in the fruit fly heads (Fig. 3); we usually analyse this compound as a spirocyclic derivative [17] but the current procedure also works quite well. The concentrations of 5 HT that were determined were in the region of those detected in locust thoracic ganglion [19], which represents an approximately similar weight of nervous tissue to that which is present in 25 *Drosophila* heads.

There were no significant differences between the concentrations of the biogenic amines detected in the four strains of *D. melanogaster*. It might have been expected that the eyeless *eya S1* strain would have shown differences in view of the absence of optic lobes but this was not the case. We have made some preliminary studies of the *N*-acetates of biogenic amines in *D. melanogaster* and have found that considerable amounts of tyramine and dopamine *N*-acetates are present in the heads.

The potential for using mutant strains of *D. melanogaster* to explore biogenic amine metabolism in invertebrates is enormous since a wide variety of mutants exist which are partially deficient in enzymes such as DOPA

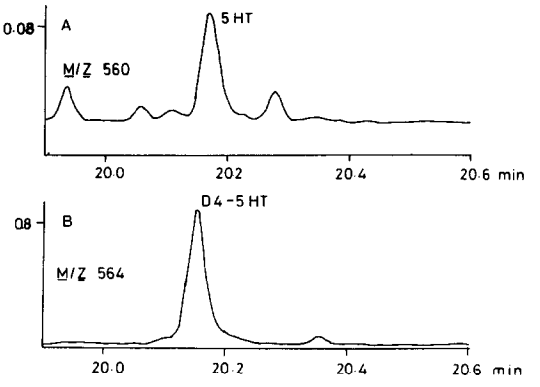

Figure 3
(A) SIM trace (*m/z* 560) showing 5 HT extracted from 25 heads of *D. melanogaster* and converted to a DTFMB–TMS derivative. (B) SIM trace (*m/z* 564) showing the ion for [$^2H_4$] 5HT; 5 ng of the amine was added to the *D. melanogaster* heads prior to extraction.

decarboxylase and tyrosinase. GC–MS seems to be the ideal technique to explore the complexities of the relationship between genetics and biogenic amine metabolism. A full understanding of biogenic amine synthesis and metabolism would facilitate the design of selective insecticides and might be extrapolated from the level of simple nervous systems in order to gain a better understanding of medical conditions such as Parkinson's disease and other neurological disorders.

Acknowledgement — We thank the University of Strathclyde for a scholarship for Ms P. Zhou.

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[Received for review 19 April 1993; revised manuscript received 24 May 1993]