

Analysis of biogenic amines and their metabolites in biological tissues and fluids by gas chromatography–negative ion chemical ionization mass spectrometry (GC–NICIMS)*

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Abstract: GC–NICIMS has been employed in the analysis of biogenic amines and their metabolites in human urine and human, bovine and porcine aqueous and vitreous humour. Several new chemical derivatization procedures have been developed in order to analyse these compounds. Concentrations of octopamines and synephrines were determined in urine from treated and untreated hypertensive subjects and normotensive individuals; there were no significant differences in concentrations of these metabolites between these groups. Human urine contained several dihydroxyphenylethylamines which have not been reported as natural metabolites before and also 5- and 6-hydroxydopamine in relatively large amounts. Aqueous and vitreous humour contained very low quantities of noradrenaline, tyramine and dopamine but measurements were inconsistent because sometimes the levels were below the limits of detection. Metabolites of a number of biogenic amines were readily detected in aqueous and vitreous humour.

Keywords: Biogenic amines; 6-hydroxydopamine; dihydroxyphenylethylamines; aqueous humour; vitreous humour; urine; GC–NICIMS.

Introduction

In earlier work we have established that trace amines such as octopamines and synephrines occur in many mammalian tissues, including sympathetic nerves [1–3]. However, we have not been able to establish any clearly defined physiological rôle for these compounds [4–6].

In earlier work we have determined concentrations of isomeric octopamines and synephrines in human urine from normotensive individuals [7]; the principal isomers from this group present in urine were *p*-synephrine and *p*-octopamine. *p*-Octopamine has high biological activity in invertebrate systems, fulfilling a similar rôle to noradrenaline [8]. Extensive work has been carried out on the measurement of adrenaline, noradrenaline and dopamine in normotensive and hypertensive subjects [9, 10] but none of this work has examined a possible rôle for octopamines and synephrines in hypertension. We decided to

apply our extraction derivatization techniques [11, 12], previously developed for the analysis of biogenic amines in vertebrate and invertebrate systems, to the analysis of trace amines in human urine from hypertensive and normotensive subjects and to those in aqueous and vitreous humour from human and bovine eye. Glaucoma (the most common cause of blindness in the West) is controlled by β -blockers [13, 14], although its aetiology is not understood. Therefore, it was of interest to determine the concentrations of biogenic amines (including noradrenaline) present in aqueous humour from glaucomatous and non-glaucomatous eye.

Experimental

Chemicals

All solvents used in extraction were HPLC grade (Rathburn Chemicals, Peebleshire, UK).

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Chemicals were obtained from the following sources: 2,3-, 2,5-, 2,6-, 3,5-, 3,6-, dimethoxybenzaldehydes; 5- and 6-hydroxydopamine, *m*- and *p*-octopamines and synephrines, *p*-hydroxyphenylacetic acid (PHPA), HVA, DOPAC and *p*-hydroxymandelic acid (PHMA); Aldrich Chemical Co., Dorset, UK. 3,5-Dinitrofluoromethylbenzoyl chloride (DTFMBCl), 3,5-dinitrofluoromethylbenzyl bromide (DTFMBzBr), *N,O*-bistrimethylsilylacetamide (BSA) and isopropyltrimethylsilyl chloride (IPDMSCl); Fluorochem, Derbyshire, UK.

Deuteriated internal standards (used to quantify compounds) were available from our earlier work [15, 16].

Dihydroxyphenylethylamines

A series of dihydroxyphenylethylamines was prepared by standard procedures [17] involving reaction of the appropriate dimethoxybenzaldehyde with nitromethane in alkaline solution followed by isolation of the resultant nitrostyrene and reduction with lithium aluminium hydride to yield a dimethoxyphenylethylamine. The methoxy groups were then removed by hydrolysis in hydrobromic acid. This work will be reported more completely at a later date.

Isopropyltrimethylsilylmethyltrifluoroacetamide (IPDMSMTFA)

This derivatization reagent was prepared according to the procedure used for the synthesis of tertiarybutyldimethylsilylmethyltrifluoroacetamide [18]. Briefly, *N*-methyltrifluoroacetamide was treated with sodium hydride in dry toluene, and this was followed by reaction of the product with isopropyltrimethylsilyl chloride. The resultant precipitate of sodium chloride was filtered off and the mixture was fractionally distilled; the IPDMSMTFA distilled from the reaction mixture at a temperature of 152°C. Its identity was checked by GC-MS and it was used for derivatization without further purification.

Aqueous humour

Samples of aqueous humour were obtained from patients undergoing surgery for lens replacement or for the alleviation of glaucoma. We have described the procedure previously [19]. Samples of porcine and bovine aqueous humour were obtained from eyes collected

from the local abattoir and were freshly analysed or frozen until required.

Urine samples

Samples of urine from hypertensive and normotensive volunteers were collected at the blood pressure clinic at Stobhill hospital. Ascorbic acid was added to samples to give a concentration of 1 mg ml⁻¹ and they were stored frozen until analysed. Before analysis, samples were normalized to creatinine using a creatinine normalization kit (Boehringer, Lewes, UK) based on complex formation with picric acid.

Sample extraction and derivatization

Biogenic amines in aqueous humour were analysed in the following way. Samples (50–200 µl) were diluted to 1 ml with potassium phosphate buffer (pH 7.4, 1 M) and appropriately deuteriated *o*-, *m*- and *p*-octopamine, *m*- and *p*-synephrine, tyramine, dopamine, adrenaline, noradrenaline and normetanephrine (1 ng of each component) were added. DTFMBCl (2 µl) was then added and the sample was sonicated to disperse the reagent; the sample was then processed as described previously [11, 12, 20]. DTFMB-IPDMS derivatives were prepared by substituting IPDMSMTFA for BSA in the final step of the derivatization.

Biogenic amines in urine were analysed in the following way. A sample of urine (equivalent to 1 mg of creatinine) was diluted to 2 ml with phosphate buffer (pH 7.4, 1 M). Samples (1 ng each) of deuteriated *o*-, *m*- and *p*-octopamines, *m*- and *p*-synephrines, noradrenaline, adrenaline and normetanephrine were added as internal standards. DTFMBCl (4 µl) was added and the sample was processed by our established procedure. Conjugated amines in urine were hydrolysed by acidifying a sample of urine (equivalent to 1 mg of creatinine) with HCl (0.5 ml, 1 M) followed by heating at 90°C for 30 min. The hydrolysed sample was diluted to 3 ml with phosphate buffer (pH 7.4, 1 M) before derivatization as described above.

Acidic metabolites of biogenic amines in aqueous humour were analysed using our established procedure, involving derivatization with DTFMBzBr [16].

GC-MS analysis

Analysis was carried out in the NICI mode as described previously [11, 12, 16]. The GC

was fitted with a BP-1 aluminium-clad fused silica column (12 m or 25 m \times 0.25 mm i.d.); helium was used as carrier gas at a head pressure of either 5 p.s.i. or 25 p.s.i. The GC was programmed as follows: 100°C (1 min) then 10°C min⁻¹ to 300°C for the amines or 140°C (1 min) then 10°C min⁻¹ to 300°C for the acidic metabolites.

Results and Discussion

Figure 1 shows a mass spectrum of the DTFMB-TMS derivative of *p*-tyramine obtained under NICI conditions. This spectrum is typical of this class of derivatives; the molecular ion and its isotope peaks carrying >60% of the ion current [11]. The retention time and molecular ion can be changed easily by altering the silylating reagent used in the final step of the derivatization procedure, e.g. if the TMS group on the DTFMB-TMS derivative of *p*-tyramine is replaced by an IPDMS group a shift in the molecular ion of 28 a.m.u. is produced. This procedure enables a highly

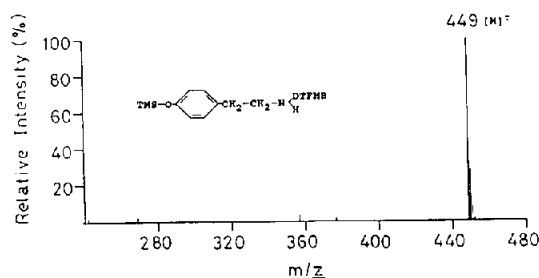


Figure 1
Mass spectrum of *p*-tyramine DTFMB-TMS derivative under NICI conditions.

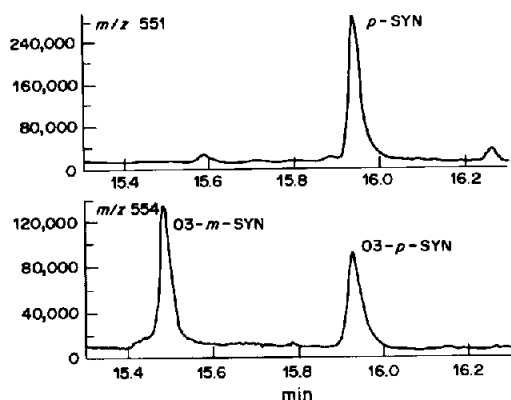


Figure 2
SIM trace of synephrines from hydrolysed urine as DTFMB-TMS derivatives (m/z 551) in comparison with a SIM trace for 5 ng of added [²H₃] internal standards (m/z 554).

specific (and sensitive) identification of metabolites to be made in biological matrices.

Figure 2 shows a selected ion trace for the DTFMB-TMS derivative of *p*-synephrine extracted from hydrolysed urine, in comparison with a SIM trace for derivatized [²H₃]*m*- and *p*-synephrine; *m*-synephrine was not consistently detected in samples and *p*-synephrine was present largely in conjugated form. It is possible that the high concentrations of *p*-synephrine observed in urine were dietary in origin; although subjects from whom samples were collected were asked to omit citrus fruit [7] from their diet for 12 h prior to sample collection. Figure 3 shows a SIM trace for *p*-octopamine extracted from urine, both as its DTFMB-TMS derivative (m/z 537) and its DTFMB-IPDMS derivative (m/z 593). The resolution of the peak for derivatized *p*-octopamine from two neighbouring interfering peaks was better in the case of the DTFMB-TMS derivative; the fact that the component affording the two interfering peaks shifted molecular weight with change of derivatization reagent in a predictable fashion is also of interest. The concentrations of octopamines and synephrines in three subject groups: normotensive (b.p. 100/49–167/106), untreated hypertensive (b.p. 155/86–189/112) and treated hypertensive (b.p. 100/49–167/106) are shown in Table 1. Although there are differences in the mean values observed for the concentrations of these compounds the large standard deviations ensure that there are no significant differences. We were not able to confirm an earlier report of the presence of *o*-octopamine in urine [7].

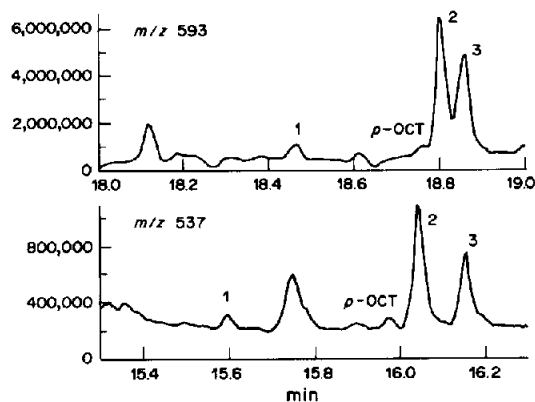


Figure 3
SIM trace of octopamines + isomers from unhydrolysed urine as DTFMB-TMS (m/z 537) and DTFMB-IPDMS (m/z 593) derivatives.

Table 1

Concentrations of octopamines and synephrines (ng creatinine mg⁻¹) in urine from normotensive, hypertensive and treated hypertensive subjects; the number of subjects from each group is shown in parentheses

Amine	Normotensive (17)		Hypertensive (5)		Treated hypertensive (8)	
	Unhydrol.	Hydrol.	Unhydrol.	Hydrol.	Unhydrol.	Hydrol.
<i>p</i> -Oct.	1.5 ± 1.1	10.9 ± 10.1	0.6 ± 0.2	5.9 ± 3.3	0.5 ± 0.4	1.9 ± 1.1
<i>p</i> -Syn.	0.5 ± 0.4	6.7 ± 12.7	0.1 ± 0.1	6.3 ± 6.8	0.1 ± 0.1	3.0 ± 4.3
<i>m</i> -Oct.	0.9 ± 0.7	1.4 ± 1.0	0.4 ± 0.4	0.9 ± 0.6	0.3 ± 0.1	0.8 ± 0.8
<i>m</i> -Syn.	0.2 ± 0.2	0.8 ± 1.6	0.2 ± 0.3	0.5 ± 0.8	0.05 ± 0.02	0.1 ± 0.1

The isomers of *p*-octopamine merited further investigation and it was observed (see Fig. 3) that the retention times of the two main isomers were between those of derivatized *p*-octopamine and dopamine (which is not shown but is present in large amounts in urine: up to 0.5 µg mg⁻¹ of creatinine). We proposed that the additional peaks, like dopamine, might be dihydroxyphenylethylamines. Synthesis of the entire series of these compounds indicated that the earlier eluted component co-chromatographed with derivatized 2,5-dihydroxyphenylethylamine (peak 2) and the later eluted component co-chromatographed with 3,5- and 2,3-dihydroxyphenylethylamines (peak 3), which were not fully resolved under the GC conditions employed. The concentrations of these compounds in urine were *ca* 30–40 ng per mg of creatinine, and this appears to be the first observation of their occurrence.

Noradrenaline was also identified and quantitatively determined in human urine, and Fig. 4 shows a SIM trace of noradrenaline, both as its DTFMB-TMS (*m/z* 625) derivative and as its DTFMB-IPDMS derivative (*m/z* 709). Again, three additional peaks appear in the traces; two of which are much larger than that of noradrenaline. The components affording two of the peaks were identified preliminarily as the noradrenaline isomers, 6-hydroxydopamine (a potent neurotoxin [21]) and 5-hydroxydopamine. So far we have been able only to obtain 2,3,4-, 3,4,5- and 3,4,6-trihydroxyphenylethylamines: although the full series of trihydroxyphenylethylamines has been synthesized previously, these compounds decompose very rapidly during cleavage of the aromatic methoxyl groups in the final step of the synthesis. Thus the third peak cannot be identified and, of course, it is not possible to confirm the identity of 6-hydroxydopamine with complete certainty until a full set of standards has been prepared.

However, certain biological data do support the likelihood of its occurrence. The early

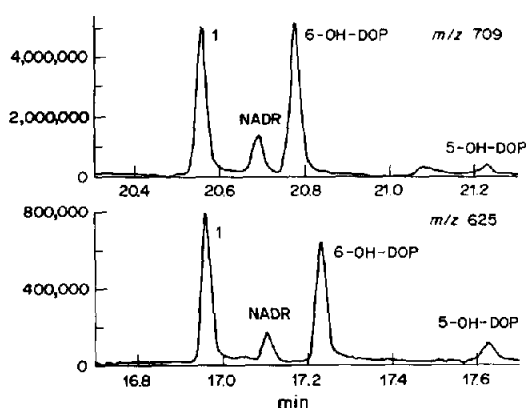


Figure 4 SIM of noradrenaline and isomers from unhydrolysed urine as DTFMB-TMS (*m/z* 625) and DTFMB-IPDMS (*m/z* 709) derivatives.

report of the occurrence of 6-hydroxydopamine in rat urine [22] was discounted subsequently since it was presumed that 6-OHDA was too unstable to occur in urine [23]; however, we have found that it does appear to be stabilized by the biological matrix. It has been found that the 2-, 5- and 6-cysteinyll adducts of DOPA do occur in urine [24] and the 5-cysteinyll adduct has been used to monitor the recurrence of melanoma [25] since it is a precursor of phacomelanin. It was discovered that an *in vitro* redox system based on ferrous-ascorbate produced dopamine hydroxylated in the 2-, 5- and 6-positions [23]. We have not obtained evidence so far for the occurrence of 6-OHDA in plasma and so it would seem likely that it might be produced via decarboxylation of 6-OHDOPA by the kidney. We are investigating these findings further since the production of 6-OHDA by the body may have considerable implications for the pathology of neurodegenerative diseases such as Parkinsonism.

Figure 5 shows a SIM trace obtained for the DTFMB-TMS derivative of noradrenaline extracted from human aqueous humour. Noradrenaline was the most consistently detected

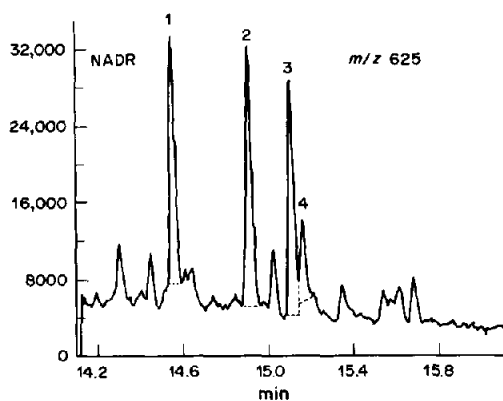


Figure 5
SIM trace of noradrenaline from human aqueous humour as its DTFMB-TMS derivatives (m/z 625).

amine in such samples. The mean concentrations of dopamine, tyramine and noradrenaline are shown in Table 2; the detection of dopamine in aqueous humour was particularly inconsistent. It has been proposed that dopamine regulates the rate of production of aqueous humour by the iris ciliary body [26] and may have a rôle in the aetiology of glaucoma. From our evidence noradrenaline may also have a rôle although the turnover of dopamine in the eye appears to be more significant than that for noradrenaline since, unlike the acidic metabolites of dopamine, acidic metabolites of noradrenaline could not be detected. The concentration of *p*-tyramine in aqueous humour was *ca* 100 times less than the concentration of *p*-hydroxyphenylacetic acid (PHPA, its acidic metabolite), indicating that it has a very high turnover rate in the eye. The concentrations of the major acidic metabolites of biogenic amines in human, bovine and porcine aqueous humour are shown in Table 3. There are particularly interesting species differences in the concentration of *p*-hydroxymandelic acid (PHMA, the metabolite of *p*-octopamine-*p*-synephrine) and also in the concentrations of dopamine metabolites, indicating that the biochemistry of such sub-

Table 2
Biogenic amines in aqueous humour from glaucomatous and non-glaucomatous eye (ng ml^{-1}); the number of subjects is shown in parentheses

Amine	COAG (8)	Cataract (10)
<i>p</i> -Tyramine	1.2 ± 0.6	3.8 ± 2.4
Dopamine	2.3 ± 0.9	1.6 ± 0.4
Noradrenaline	1.3 ± 0.6	2.1 ± 0.8

Table 3
Acidic metabolites of biogenic amines from human, bovine and porcine eye (ng ml^{-1}); the number of subjects is shown in parentheses

Metabolite	Human (66)	Bovine (6)	Porcine (3)
PHPA	71 ± 12	51 ± 14	21 ± 13
HVA	10 ± 2	—*	—*
DOPAC	2 ± 0.3	6 ± 2.4	11 ± 8
PHMA	16 ± 2	2 ± 0.5	8 ± 2

* $<0.5 \text{ ng ml}^{-1}$.

stances in the eye is more subtle than previously thought.

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