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A Simple Procedure for the Assay of Brain Biogenic Amines by Selected-ion Monitoring: Its Application to the Elucidation of the Mechanism of Prolactin Release Induced by 3-Iodo-L-tyrosine

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Abstract

A simple method for the assay of brain biogenic amines by selected-ion monitoring was applied to examination of the effects of 3-iodo-L-tyrosine on the hypothalamic–median eminence concentrations of dopamine, noradrenaline and serotonin in the rat. Thirty minutes after its administration iodotyrosine (50 mg/kg) caused a highly significant ($P < 0.0005$) rise in serum prolactin and a highly significant ($P < 0.0025$) fall in the concentration of dopamine in the hypothalamus and median eminence where the levels reached 50% of control levels. Less marked but significant falls were also observed in the hypothalmic ($P < 0.05$) and median eminence ($P < 0.0025$) concentrations of noradrenaline after iodotyrosine administration. Serotonin concentration was significantly reduced ($P < 0.025$) in the median eminence but not in the hypothalamus after iodotyrosine administration.

These findings suggest that iodotyrosine exerts its prolactin stimulating effect by blockage of dopamine synthesis rather than by receptor blockade.

Introduction

It is generally accepted that the central nervous system exerts control over the release of pituitary hormones via biogenic monoamines operating at the level of the hypothalamus. The biogenic amines thought primarily to be involved in these mechanisms are the catecholamines dopamine and noradrenaline, and the indoleamine serotonin. However, the precise roles of the monoamines in specific neuroendocrine mechanisms remain controversial and unresolved (Smythe 1977). A lot of the data used to formulate hypotheses about the roles of the brain monoamines in the release of pituitary hormones have been derived by using drugs to alter the activity and concentrations of the brain monoamines and then observing the resultant effects on pituitary hormone release. The results of such studies are open to different interpretations due to the lack of specificity of drugs used to change monoamine activity. There have been relatively few studies which have investigated the dynamic relationship between endogenous brain monoamine levels and/or turnover and physiological changes in pituitary hormone secretion. Likewise there have been few studies in which brain biogenic amine concentrations and neuroendocrine activity have been correlated following drug administration. The lack of such studies has been due to the unavailability of highly sensitive methods for the precise assay of specific biogenic amines. The development of sensitive radio-enzymic methods to measure tissue levels of catecholamines (Coyle and Henry 1973) has enabled physiological studies of the kind referred to above to be initiated (Advis *et al.* 1978) but this method is not absolutely specific and cross-contamination remains a problem (Costa *et al.* 1974).

The most significant recent advance in techniques for the measurement of biogenic amines in brain nuclei is that of quantitative mass spectrometry or, more specifically, selected-ion monitoring (SIM) (Costa *et al.* 1974). This technique employs a gas chromatograph interfaced to a mass spectrometer (GC/MS), the detection system of which can be simultaneously focussed on one or more specific fragment ions derived from the compound(s) under investigation.

SIM is one to two orders of magnitude more sensitive than other methods used to measure monoamines (Costa *et al.* 1974) and is highly specific. The task of simultaneously detecting specific fragment ions is most conveniently done using a computer interfaced to the GC/MS instrumentation; several such systems are now commercially available. SIM is made an even more attractive technique for the assay of biogenic amines if deuterated analogues of the amines are used as internal standards to optimally control for losses during the extraction and analytical procedure (Beck *et al.* 1977). While elaborate methods have been developed in the attempt to separate the biogenic amines from each other prior to assay (Atack and Magnusson 1978) this necessity is obviated in SIM by the integral use of gas chromatography. The method, however, requires that the monoamines be converted to volatile derivatives prior to assay.

The aim of this investigation was to develop a simple, flexible procedure for the extraction and derivatization of brain monoamines which would be suitable for their assay by SIM using deuterated analogues as internal standards.

The method was applied to the measurement of hypothalamic and median eminance biogenic amine concentrations in order to elucidate the mechanism whereby 3-iodo-L-tyrosine (MIT) induces pituitary prolactin release (Smythe *et al.* 1974, 1975). While MIT is an inhibitor of tyrosine hydroxylase (Udenfriend *et al.* 1965) it is rapidly cleared and must be given to animals repeatedly in high doses for 6 h to achieve a significant depletion of brain noradrenaline (NA) (Spector *et al.* 1965). On the basis of these findings with respect to NA and the rapidity with which MIT causes the release of prolactin, we have suggested previously that this compound acts in this situation to block dopamine–receptor interactions rather than by blockade of catecholamine synthesis (Smythe *et al.* 1974). It was anticipated that the use of SIM could help decide which of these two possibilities was the more likely.

Materials and Methods

Instrumentation

The studies were carried out using a Hewlett–Packard 5993A GC/MS data system. For these studies all glass gas chromatograph columns (870 by 2 mm) packed with either 3% OV-101 on Chromosorb W (Applied Science Labs Inc.) or 3% SP2100 on 100/120 Supelcoport (Supelco Inc.) were used. Helium carrier gas (30 ml/min flow rate) was used throughout and a membrane separator was used at the GC/MS interface. All the data reported here were obtained with the gas chromatograph oven temperature programmed to rise from 154°C (after 30 s) to 180°C at 30°/min. An injection port temperature of 220°C was used. Mass spectra of the pure monoamine and deuterated analogue derivatives were obtained using the instrument in scanning mode, and analytical data were obtained using the SIM mode which measures the intensity at the most intense 0·1 atomic mass unit point for each of the mass peaks chosen during each scan. With this system up to five groups each of four fragment ion peaks can be preselected prior to the start of the SIM run. These groups of ions can be automatically scanned by preselecting the group starting time or they may be manually switched to at any time during the run.

The fragment ions selected for analysis of the derivatized amines and retention times are given in Table 1. The ions selected are not the molecular ions but are those due to McLafferty rearrangements and which were usually the base peaks in the mass spectra.

Standards

The results reported in this study are uncorrected and refer to the free bases of the following compounds which were dried *in vacuo* prior to use: dopamine hydrochloride (Calbiochem, Carlingford, N.S.W.); L-noradrenaline (Sigma Chemical Co., Mo., U.S.A.); serotonin (5-hydroxytryptamine, 5-HT) (creatinine sulfate complex, Sigma Chemical Co.); 2,5,6-trideuterodopamine (d_3-dopamine, d_3-DA) prepared by deuterium exchange from dopamine hydrochloride by Dr R. Vining, Garvan Institute (unpublished data); α,β,β-trideuteronorepinephrine bitartrate (d_3-NA) (ICN Pharmaceuticals, U.S.A.); $\alpha,\alpha,\beta,\beta$-tetradeutero-5-hydroxytryptamine creatinine sulfate complex (d_4-5-HT) (Dr G. A. W. Milne, NIH, Bethesda, Md, U.S.A. (see Shaw *et al.* 1976).

The deuterated standards were determined to be chromatographically pure by analysing their derivatives on both scanning and SIM mode. Isotopic purity was estimated by simultaneously monitoring the molecular ions for species of zero level of deuteration up to maximum deuteration (i.e. d_0[M+], d_1[M+], d_2[M+] etc.).

Table 1. Ions monitored for the derivatized monoamines

Amine	Selected ion m/z		Approx. retention time (min)
	Trifluoroacetyl	Pentafluoropropionyl	
DA	328	428	1·0
d_3-DA	331	431	1·0
NA	440	590	0·9
d_3-NA	442	592	0·9
5-HT	351	451	2·7
d_4-5-HT	354	454	2·7

Animals

Normal male rats of the Wistar strain aged 50 days were used in this study. The animals were injected intraperitoneally with 0·5 ml physiological saline (controls) or with a suspension of MIT (3-iodo-L-tyrosine, Sigma Chemical Co., 50 mg/kg) in 0·5 ml saline. Then 30 min after injection the animals were decapitated and blood was collected for serum prolactin determination. The brains were rapidly removed, and hypothalamic and median eminence sections taken, weighed and immediately subjected to the extraction procedure. The hypothalamus was dissected between levels A 3·0 mm and A 5·0 mm (Konig and Klippel 1963) and the extreme basal portion of this fragment, containing part of the arcuate nucleus (Ungerstedt 1971), was dissected as the median eminence. The mean wet weight of median eminence samples was 10 mg and the mean wet weight of the remaining hypothalamic samples was 30 mg.

Extraction Procedure

Tissue sections were mechanically homogenized (Ultra-Turrax) in a solution of 5 M formic acid in n-butanol (20%, 1 ml) which contained known amounts of d_3-DA, d_3-NA and d_4-5-HT. The amounts of deuterated amines routinely added to each tissue sample in this study were 200 pmol DA, 350 pmol NA and 500 pmol 5-HT. The mixture was then centrifuged for 30 min at 1500 *g*, 4°C. The supernatant solutions were then decanted into clean test tubes containing heptane (2 ml) and 5 M formic acid (200 μl), vortexed for 15 s and centrifuged at 1500 *g* for 1 min.

The aqueous phase which contained the monoamines was then transferred to 1 ml Reacti-vials (Pierce Chemical Co., Ill., U.S.A.). The samples are quite stable at this stage and may be frozen for long storage or refrigerated at 4°C for several days prior to assay. Prior to derivatization the aqueous formic acid solution containing the monoamines was evaporated to dryness either by using a stream of dry nitrogen or a Speed-Vac concentrator (Savant Instrument Inc., N.Y., U.S.A.).

Derivatization

Trifluoroacetyl derivatives were prepared by reacting the dried monoamine residue with trifluoroacetic anhydride (TFAA; 100 μl, Pierce Chemical Co.) in dry acetonitrile (50 μl) for 1 h at 60°C.

Pentafluoropropionyl derivatives were prepared by reacting the dried monoamine residue with pentafluoropropionic anhydride (PFPA; 100 μl, Pierce Chemical Co.) in acetonitrile (50 μl) for either 30 min at 60°C or overnight at 0–4°C.

Prior to injection of samples into the GC/MS system, solvent and excess reagent were removed under a stream of dry nitrogen and the residue dissolved in 10 μl of a 1% solution of the anhydride used for derivatization (TFAA or PFPA) in dry ethyl acetate.

Calibration of Deuterated Standards

The amount of the deuterated standards added to each tissue sample was calibrated for each assay by adding this amount to an equivalent aliquot from a solution of accurately weighed non-deuterated DA, NA and 5-HT. This mixture (in replicate) was dried, derivatized and analysed in the GC/MS system using the SIM mode. The amount of the deuterated amines in the mixture was calculated by comparing the peak areas for the deuterated ions with the equivalent ion from the non-deuterated amines. The accuracy of this procedure was unaffected by the amount of non-deuterated amines present in a mixture when any or all of the non-deuterated components were varied over a concentration range from 300 fmol/μl to 3 mmol/μl.

The measurement of endogenous amine concentration was carried out by comparison of fragment ion peak areas (SIM) with those of the corresponding deuterated ions. All results are expressed as picomoles per milligram of tissue, wet weight.

Radioimmunoassay

Serum levels of prolactin were measured by radioimmunoassay using materials supplied by Dr A. F. Parlow (NIAMDD, Rat Pituitary Hormone Program).

Statistics

Statistical analyses were carried out using Students' *t*-test.

Results

The isotopic purity of the deuterated standards was very high and less than 0·1% of non-deuterated species were detected. Deuterated species were determined to be (as moles per 100 mol) 93·5 d_3, 6·1 d_2 and 0·4 d_1 for d_3-DA; 94·4 d_3 and 5·6 d_2 for d_3-NA; and 94·7 d_4 and 5·3 d_3 for d_4-5-HT. No detectable exchange of deuterium in these compounds occurred over 12 months of frozen storage in dilute HCl or formic acid.

The duration of the derivatization reaction could be conveniently extended when necessary by carrying out the reaction at 0–4°C. At these temperatures the PFPA reaction mixtures were stable for at least 3 days although significant losses occurred after 20 days. On the other hand, the TFA reaction mixture remained stable for at least 20 days. No significant exchange of hydrogen for deuterium in the deuterated standards occurred during this time with either anhydride.

The extraction of endogenous DA, NA and 5-HT from brain tissue was found to be efficient and recoveries, which were estimated from the fragment ion peak areas of the added deuterated standards, routinely exceeded 90%. Table 2 shows the excellent reproducibility obtained using this technique. Under the conditions used in this study a sensitivity of less than 100 pg (50 fmol/mg tissue) was attainable but rarely necessary.

The effect of the administration of MIT on the concentrations of DA, NA and 5-HT in the hypothalamus and median eminence is shown in Figs 1 and 2. In the

hypothalamus MIT caused a highly significant decrease in the concentration of DA ($P < 0{\cdot}0025$) to 67% of controls and a small but significant decrease in the concen-

Table 2. Reproducibility of monoamine assay by SIM
n, Number of times sample assayed

Amine	*n*	Mean assay ± s.d. (pmol)	Coefficient of variation (%)
	(*a*) Within-assay variation[A]		
Dopamine	9	142·5 ± 3·3	2·3
Noradrenaline	9	42·8 ± 1·5	3·4
Serotonin	9	25·0 ± 0·6	2·3
	(*b*) Between-assay variation[B]		
Dopamine	10	65·3 ± 2·5	3·8
Noradrenaline	10	36·1 ± 1·2	3·9
Serotonin	10	21·6 ± 0·8	3·7

[A] Data in (*a*) and (*b*) obtained using different samples.
[B] Samples stored in 0·25 M formic acid between assays.

tration of NA ($P < 0{\cdot}05$). No significant effect was observed for 5-HT. In the median eminence, however, the effects were more pronounced, both DA and NA concentrations being very significantly lower ($P < 0{\cdot}0025$) after MIT administration, the con-

Fig. 1

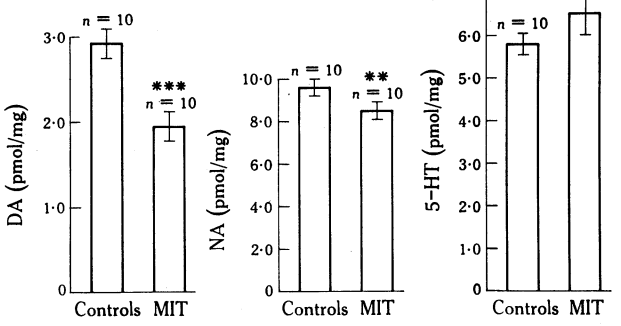
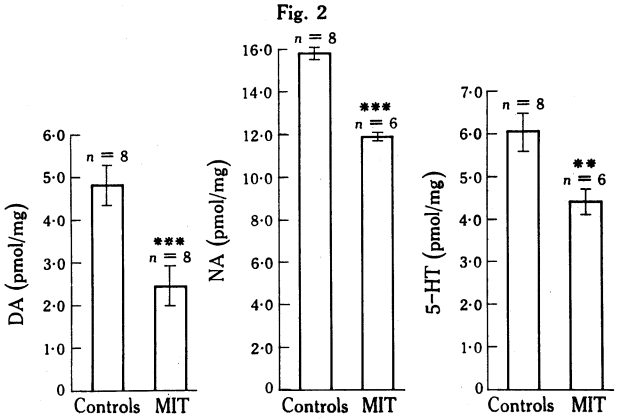


Fig. 2



Figs 1 and 2. Hypothalamic (Fig. 1) and median eminence (Fig. 2) concentrations (wet weight) of DA, NA and 5-HT in saline-treated (controls) and MIT-treated (50 mg/kg) rats 30 min after administration. Means ± s.e.m. are shown. *n*, Number of animals sampled. In (*a*), * not significantly different from controls; ** $P < 0{\cdot}05$ *v.* controls; *** $P < 0{\cdot}0025$ *v.* controls. In (*b*), ** $P < 0{\cdot}025$ *v.* controls; *** $P < 0{\cdot}0025$ *v.* controls.

centrations of DA and NA falling to approximately 50 and 75% of controls respectively. A significant decrease in 5-HT concentration ($P < 0{\cdot}025$) was observed in the median eminence.

The dose of MIT used in this study (50 mg/kg) was half that given to rats previously (Smythe *et al.* 1974) but it caused a similar, highly significant ($P < 0{\cdot}0005$) increase in serum prolactin concentration from $15{\cdot}5 \pm 2{\cdot}6$ ng/ml (mean $\pm$ s.e.) in the control group to $68{\cdot}7 \pm 12{\cdot}0$ ng/ml in the MIT-treated group.

Discussion

The method used to measure brain tissue concentrations of the biogenic amines in this study is simple and flexible. The use, as standards, of deuterated analogues which possess virtually identical physical and chemical properties with the endogenous amines avoids the necessity of critical allowance for and measurement of losses during extraction, derivatization and measurement. Once the tissues are homogenized with deuterated standards the mixtures are relatively stable. Once the amines are isolated into the aqueous formic acid they can be kept in this condition for extended periods despite the very low concentrations present. In this regard formic acid, which possesses reducing properties, was found superior to the traditional hydrochloric acid. The fact that there is no need for samples to be assayed immediately they are derivatized using this method gives it added flexibility. Furthermore, the method is readily adaptable to the measurement of plasma biogenic amines by SIM. The results of this study demonstrate that SIM methods are eminently suitable for the assay of brain biogenic amines. The method has allowed the clear demonstration that MIT within 30 min acts to cause a marked decrease in DA concentration in both hypothalamic and median eminence tissue.

The observed effect is almost certainly due to blockade of synthesis of DA by tyrosine hydroxylase inhibition (Udenfriend *et al.* 1965). This is substantiated by the finding of a significant reduction in NA concentrations also which was aided by the rather small within-group variation in observed NA concentration. The effects on NA were less marked than those seen for DA which presumably reflects a more rapid turnover of DA. Previous studies (Spector *et al.* 1965) using fluorometric methods did not measure DA but found a significant reduction of brain NA only after repeated high doses of MIT to rats, thus demonstrating the advantages of the current method.

The finding that MIT caused a significant decrease in the concentration of 5-HT in the median eminence was unexpected but has been confirmed by us in more recent experiments. The reason for this is not clear at present but the result may indicate a dependence of 5-HT synthesis on DA in certain nuclei. This question will be further investigated.

In conclusion, a simple method for the assay of biogenic amines has been developed which is capable of demonstrating small and rapid changes in brain monoamine concentrations. The data obtained enable a decision to now be made about the mechanism of action of MIT on prolactin release. We propose that MIT effects its quick and marked stimulation of prolactin secretion by a rapid depletion of hypothalamic and median eminence DA due to synthesis blockade.

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