MASS SPECTROMETRY OF CRUDE BIOLOGICAL EXTRACTS

ABSOLUTE QUANTITATIVE DETECTION OF METABOLITES AT THE SUBMICROGRAM LEVEL

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SUMMARY

　　In this paper a new method of unambiguous and ultramicro analysis of metabolites in crude biological extracts is described. The technique—the so-called "integrated ion-current technique"—involves a large double-focussing mass spectrometer; the ion current at a particular m/e value is recorded. All other ions are excluded; the ion current recorded may be quantitated. Virtually any metabolite is amenable to analysis using this technique and quantitation is possible in the range 10^{-12} to 10^{-5} g. p-Tyramine, an interesting biogenic amine, has been positively identified in rat brain for the first time and its quantitative regional distribution has been established.

INTRODUCTION

　　It is difficult to be certain of the identity of a substance simply after the demonstration of isographic behaviour between the unknown and a suspected synthetic standard in several different solvent systems. Of course if derivatives of the unknown and the suspected standard exhibit isographic behaviour and also possess similar gross physicochemical characteristics the certainty is enhanced but still not necessarily absolute. It has recently been shown[1] that even though paper, thin-layer and gas chromatographic evidence indicated identity between an unknown and a standard, they were in fact different. These difficulties of identification are often compounded for the biochemist because the unknown is frequently present in small amounts in an extract exhibiting a wide spectrum of metabolites; further fractionation and purification often produces artefacts which seem to bear a striking resemblance to the unknown in everything but structure. It has become a common, but not yet standard, practice to list additional analytical data such as ion-exchange and gas chromatographic properties and visible, UV, IR and mass spectra. Conventional spectral data

are usually not much help unless the unknown is present in relatively large uncontaminated amounts. Low-resolution mass spectrometry is frequently coupled to the effluent from a gas–liquid column, high-resolution devices usually present unambiguous data as a consequence of their precise mass-measuring facility.

We describe here the use of a new technique, the so-called integrated ion current (IIC) technique which seems to possess many advantages in the qualitative and quantitative analysis of small amounts of materials present in complex extracts. Preliminary details have already been published[2].

The presence and concentration of p-tyramine in urinary and rat brain extracts has been selected for a demonstration of the use of the technique since this substance seems of interest in various neurological[3-6] conditions and in addition other quantitative techniques were available for confirmation and comparison.

MATERIALS AND METHODS

Chromatographic analysis of urinary p-tyramine

The amount of p-tyramine present in a urinary phenolic amine extract was determined after paper chromatographic separation and scanning of the 1-nitroso-2-naphthol fluorophore. This technique has been described in detail[7]. The overall efficiency of extraction is about 70%, quantitation is possible in the 0.5 to 10 μg range with an error of $\pm 5.2\%$ (95% fiducial limits). The time taken to effect a complete analysis is about 18 h, 14 of which is overnight chromatographic development.

Chromatographic analysis of p-tyramine from brain extracts

A preliminary account of this technique has recently been given[2,8]. Briefly, rats are stunned, decapitated and their brains removed and placed in ice-cold saline solution. Whole brains or certain dissected areas are then washed in ice-cold saline solution and homogenised in 0.4 N HClO$_4$. After centrifugation the supernatant is neutralised with 1 N NaOH. The phenolic amine fraction is isolated as described by KAKIMOTO AND ARMSTRONG[9] and after trituration in 70% ethanol the whole extract is transferred to a thin layer of silica gel (Kodak 6061) and separated in butanol–acetic acid–water (4:1:1). The p-tyramine zone, as indicated by markers run alongside, is eluted (90% methanol), dried and dissolved in 100 μl of 0.1 M NaHCO$_3$. Dansylation is achieved by adding 100 μl of dansyl reagent (1-dimethylamino-naphthalene-5-sulphonyl chloride) (1 mg/ml in acetone) and leaving overnight at room temperature. Sodium bicarbonate is removed by centrifugation after adding 4 vol. of acetone. The supernatant is dried, redissolved in benzene–acetic acid (99:1) and then separated on a silica gel thin layer (Kodak 6061) in the solvent system chloroform–butyl acetate (5:1)[10]. The dansyl tyramine zone is eluted in benzene–acetic acid (99:1) and re-run in ethyl acetate–cyclohexane (2:3)[10]. The dansyl tyramine derivative is then finally eluted in benzene–acetic acid (99:1)[10] and the amount of fluorescence measured (activation 340 nm, emission 510 nm). A blank reading is obtained by subjecting similar areas from empty plates to the whole procedure. The overall efficiency of extraction, separation and analysis is about 54%, quantitation in the 5 to 100 ng range is possible with an error of $\pm 10.2\%$ (95% fiducial limits). The time taken to effect a complete analysis involves three working days.

Integrated ion current analysis of p-tyramine

A preliminary report on this technique has been published[2]; essentially the procedure is as follows. Samples (5 μl) of the phenolic amine extracts, dissolved in 70% ethanol or ethanol–ammonia (70:30), are evaporated from the probe, into the ion source of the mass spectrometer at a temperature of 220° (MS 9, Associated Electrical Industries Ltd., Manchester, U.K.). During evaporation ion currents are recorded at the selected m/e values 137 or 108, representing the molecule ion peak $C_8H_{11}ON^+$ or the base peak $C_7H_8O^+$ of p-tyramine, respectively. The overall efficiency of extraction of p-tyramine added to brain homogenates and processed by this technique is better than 90%; quantitation is possible in the 10^{-12} to 10^{-5} g range with an error of $\pm5\%$ (95% fiducial limits). The time taken to effect a complete analysis (assuming immediate access to the mass spectrometer and an experienced technician) is about 2 h.
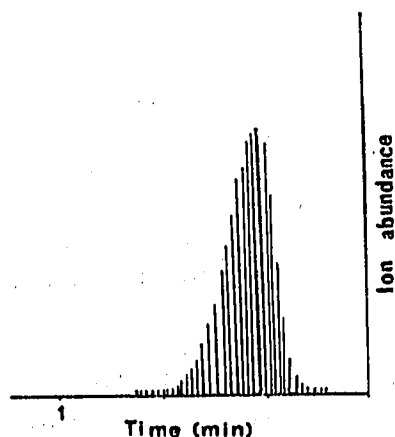

Fig. 1. Record of the ion current at m/e value 137 during evaporation at 220° of 1 ng of p-tyramine.

RESULTS

The shape of the envelope enclosing the recorded ion currents at an m/e value of 137 is illustrated in Fig. 1. In this particular example 1 ng of p-tyramine (free base) was evaporated from the probe at 220°. The area under the curve is proportional to the concentration of p-tyramine present on the probe in all cases if the operating conditions are identical; the useful working range is 10^{-12} to 10^{-5} g.

When a sample of a urinary phenolic amine extract dissolved in 70% ethanol was analysed, the record of the ion current at m/e 108 was abnormal (see Fig. 2). This shape changed to a more conventional one when p-tyramine (free base) was added to the urine extract before analysis, even though the record was still to some extent elongated. At best, that is when large quantities of p-tyramine had been added to the urine before extraction, the shape of the resultant curve was midway between those illustrated in Figs. 2 and 3. Such behaviour suggested that the extracted tyramine was complexed in a way which limited its ability to evaporate from the probe. The technique used to extract the phenolic amine fraction from urine[9] involves absorption of the phenolic amines on an ion-exchange resin followed by washing with aqueous sodium acetate and elution with alcoholic ammonia. It seems quite possible, there-
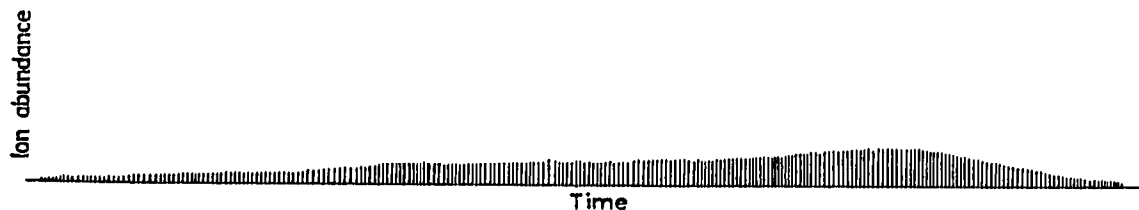
Fig. 2. Record of the ion current at m/e value 108 during evaporation at 220° of a urine phenolic amine extract (5 μl) dissolved in 70% ethanol.

fore, that the amines exist in the final dried extract as the acetate salt (such a phenomenon would not be observed if the subsequent analytical steps involved chromatographic separation). Trituration of the phenolic amine extract in ethanol–ammonia (70:30) overcame this problem and the subsequent ion current records exhibited the expected Gaussian distribution (see Fig. 3).

The concentration of p-tyramine in urine as obtained by analysis using the IIC technique agreed well with the chromatographic method based on the 1-nitroso-2-naphthol fluorophore even though in this particular analysis the ion current curves were elongated and peak limits were applied. Supplementation of the urine samples with increasing quantities of p-tyramine (free base) yielded the expected analytical data after extraction and IIC analysis (see Table I).

When the IIC technique was extended to the analysis of extracts from whole rat brain and rat brain regions, dissolved in ethanol–ammonia (70:30), the ion current curve obtained was complicated slightly by the appearance of a double peak in the records obtained both at m/e values 108 and 137 (see Fig. 4). The two peaks are separate, however, and precise mass measurement has allocated at m/e 108 the values 108·0575, $C_7H_8O^+$ from p-tyramine, and 108·0939, $C_8H_{12}^+$, and at m/e 137 the values 137·0841, $C_8H_{11}ON^+$ from p-tyramine, and 137.1330, $C_{10}H_{17}^+$. The hydrocarbon moiety probably arises from a lipid source during the extraction procedure. The fact that these two peaks produce a record as shown in Fig. 4 means that p-tyramine can be resolved and quantitative data obtained by reference to a calibration curve in the usual way. Quantitative data could be obtained, at the same time, for the hydrocarbon peak if this was deemed necessary or desirable.
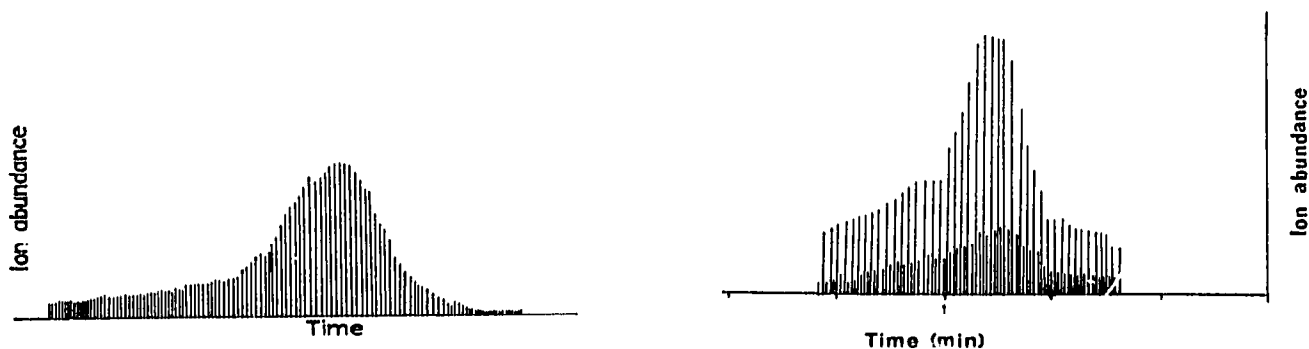

Fig. 3. Record of the ion current at m/e value 108 during evaporation at 220° of a urine phenolic amine extract (5 μl) dissolved in ethanol–ammonia (70:30).

Fig. 4. Record of the ion current at m/e value 108 during evaporation at 220° of a rat brain phenolic amine extract (5 μl) dissolved in ethanol–ammonia (70:30).

TABLE I

ANALYSIS OF p-TYRAMINE IN A URINARY EXTRACT

The phenolic amine extract from 400 ml of urine in alcoholic ammonia was divided into six aliquot parts. Samples C–F were supplemented with p-tyramine (free base) as shown. After rotary evaporation each sample was dissolved in 250 μl of 70% ethanol and because the ion current curves under these conditions were non-Gaussian (see Fig. 2) limits were applied in order to obtain quantitative data.

Sample	IIC analysis		Expected value
	$ng/5\ \mu l$	$\mu g\ in\ sample$	
A	50	2.50	2.64[a]
B	57	2.85	
C + 5 μg	130	6.50	7.64
D + 10 μg	250	12.50	12.64
E + 25 μg	510	25.50	27.64
F + 50 μg	1140	57.00	52.64

[a] This value is based on the average value obtained from several estimations using the fluorimetric chromatographic technique (see text).

A comparison of the p-tyramine content of whole rat brain and certain regions (see Table II) using the IIC technique is compared with some values obtained using the fluorimetric dansyl technique based on chromatographic separations.

DISCUSSION

The quantitative data on the p-tyramine content in urinary and tissue extracts as illustrated in this paper using the new IIC technique and chromatographic procedures are very similar. It is a fact, however, that although the chromatographic procedures chosen for these analyses were fairly specific, being based in the one case on selective absorption, paper chromatographic separation and the formation of a fluorophore and in the other case on selective absorption, separation, preparation of a fluorescent derivative and two further thin-layer separations, absolute identifi-

TABLE II

REGIONAL DISTRIBUTION OF p-TYRAMINE IN RAT BRAIN

Brain area	IIC technique		Chromatographic technique
	$ng\ p\text{-}tyramine$	$ng\ p\text{-}tyramine$ $per\ g\ tissue$	$ng\ p\text{-}tyramine$ $per\ g\ tissue$
Whole brain[a]	21.8	221	192 ± 24[c]
Caudate nucleus[b]	5.1	587	—
Hypothalamus[b]	7.3	1719	—
Cerebellum[b]	7.7	280	—
Stem[b]	4.6	178	—
Rest[b]	7.4	54.6	—

[a] Average of four estimations.
[b] Average of three estimations.
[c] Eight estimations (standard error).

cation was lacking. In many cases it is not always possible to select chromatographic procedures which will yield the selectivity and sensitivity required. The IIC technique offers not only certainty of identification but also supreme sensitivity[11] and a considerable economy in time.

The fact that a double envelope was seen in the IIC analysis of brain extracts is a good illustration of the power of the method, *viz.* the fragments $C_7H_8O^+$ and $C_8H_{12}^+$ at an *m/e* value 108 differed by only 464 p.p.m. and yet they were clearly separated. Their atomic constitution was easily obtained by precise mass measurement. The likelihood of two or more fragments occurring at any particular *m/e* value is not high but when it does occur an absolute constitution can be assigned after precise mass measurement. Alternatively such a situation can often be avoided by using a different *m/e* value or else by altering one of the pairs by deuterium exchange labelling or the preparation of a derivative. It is even possible in susceptible cases to use the fine structure exhibited by the shape of the ion current envelope to distinguish between stereochemical isomers[12]. In the case of *ortho*, *meta* and *para* isomers of a molecule, as is the case for hydroxyphenylethylamine, the ratio of the ion currents at certain *m/e* values is different. A "scan" before IIC analysis reveals which molecule is present and small differences in the rates of evaporation of different components can reveal how much of each is present.

There seems little doubt that the IIC technique is the method of choice in the analysis of small amounts of materials present in complex biological extracts. The only snag seems to be that the necessary mass spectrometer installation is large and expensive and requires a skilled technician for optimum operation.

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