APPLICATION OF MASS FRAGMENTOGRAPHY TO THE QUANTITATION OF ENDOGENOUS CATECHOLAMINES

STEPHEN H. KOSLOW
Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Saint Elizabeths Hospital, Washington, D.C. 20032, U.S.A.

THE USE of quantitative gas chromatography-mass spectrometry (GC-MS) for the measurement of punative neurotransmitters (KOSLOW *et al.*, 1972; CATTABENI *et al.*, 1972) offers many advantages over the presently used methods. It can be applied to the simultaneous measurement of endogenous norepinephrine (NE), epinephrine (E) and dopamine (DA) over a wide range of tissue samples. The preparation of the samples is fast and efficient and the analysis offers a high degree of specificity with sensitivity greater than 10^{-15} mole. With this high sensitivity as will be described in this paper, it is possible to measure the catecholamine content of 30 μm sections of neuronal tissue.

The GC serves to resolve the various neurotransmitters from each other, while the MS is used for quantitation by measuring the ion density of specific characteristic fragments (mass-to-charge, m/e) of each compound. Prior to analysis it is necessary to prepare catecholamine derivatives with the appropriate vapour pressure for GC. This is done by reacting the catecholamines with pentafluoropropionic anhydride (PFPA) resulting in the acylation of the phenoxy and β-hydroxy and primary and secondary amino groups. Using an LKB 9000 GC-MS the structures of the derivitised (PFP) catecholamines were confirmed by mass spectral analysis. Figure 1 shows a partial mass spectra of the three catecholamine-PFP derivatives (complete spectra have been published, KOSLOW *et al.*, 1972).

For quantitation, the ion density of the most abundant fragment (base peak, 100 per cent relative intensity) is recorded at the compound's retention time (Fig. 2, Table 1). α-Methylnorepinephrine (αMNE) and α-methyldopamine (αMDA) are included as internal standards and fulfill all the requirements of an internal standard (KOSLOW *et al.*, 1972); αMNE is the internal standard for NE and E, while αMDA is the internal standard for DA. For analysis tissues samples are homogenised in 0·1 M formic acid (50 mM ascorbate) and an aliquot of the supernatant is dried under nitrogen with an exact concentration of the internal standard. Concomitantly, authentic standards (over the appropriate concentration range) containing the same concentration of internal standards are prepared. After reacting the dried samples with 100 μl PFPA, 20 μl ethylacetate (30 min), the sample is dried under nitrogen and reconstituted in 10 μl ethylacetate. Usually, 2 μg of this solution is injected into the GC port of the GC-MS. A typical recording is shown in Fig. 2.

The ratio formed by dividing the peak height (or area) of the catecholamine by the peak height (or area) of the appropriate internal standard is plotted against the known concentration of catecholamine reacted. In this way the linear response obtained is used to quantitate endogenous catecholamine levels.

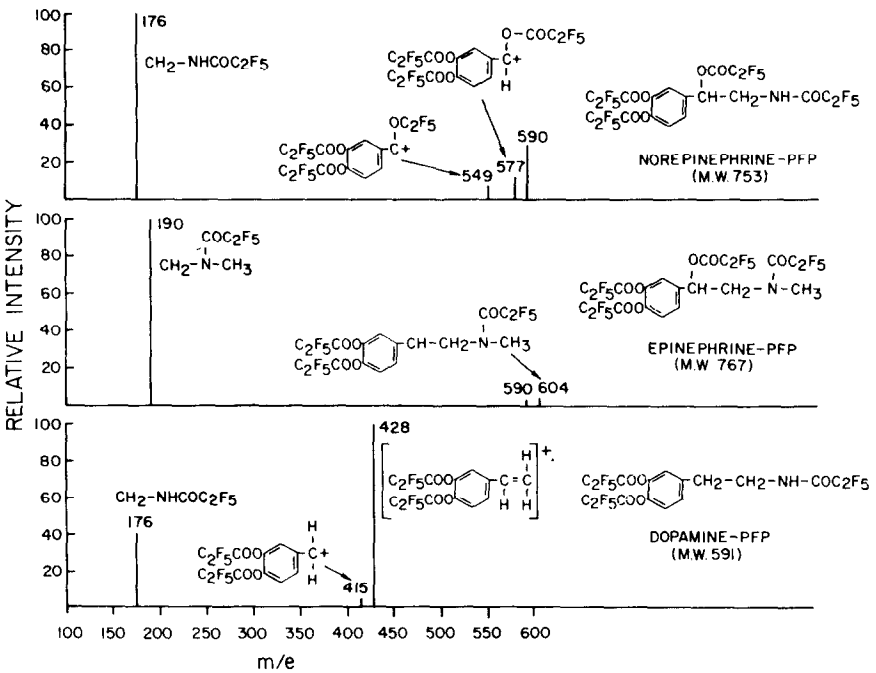

FIG. 1.—Partial mass spectra of catecholamines acylated with pentafluoropropionic anhydride. The base peaks (100 per cent relative abundance) are monitored for quantitation and the other fragments whose theoretical structures are shown are monitored for multiple ion detection (MID) for specificity. The fragment at m/e 590 cannot be monitored for MID since it is present for both norepinephrine and epinephrine and both of these compounds have the same retention time.

TABLE 1. GAS CHROMATOGRAPHIC (GC) RETENTION TIME AND POSITIVE IONS MONITORED FOR QUANTITATION AND MULTIPLE ION DETECTION (MID) ANALYSIS OF ACYLATED CATECHOLAMINE DERIVATIVES

PFP amine derivatives	GC retention times (min)	Quantitation m/e	Multiple ion detection m/e (%)*	Fragment ratio
α-Methyl-nor-epinephrine	2·08	190		
Norepinephrine	2·83	190	577 (12)	
			549 (7)	1·7
Epinephrine	2·83	176	190 (100)	
			604 (4)	25
α-Methyl dopamine	4·36	442		
Dopamine	5·83	428	428 (100)	
			415 (5)	20

* (%) refers to the relative intensity of the fragment compared to the most abundant (base) peak which is 100%. MID is not done on internal standards, tissue samples are however, processed without internal standards to insure the absence of any peak at this GC time and m/e due to "biological background".

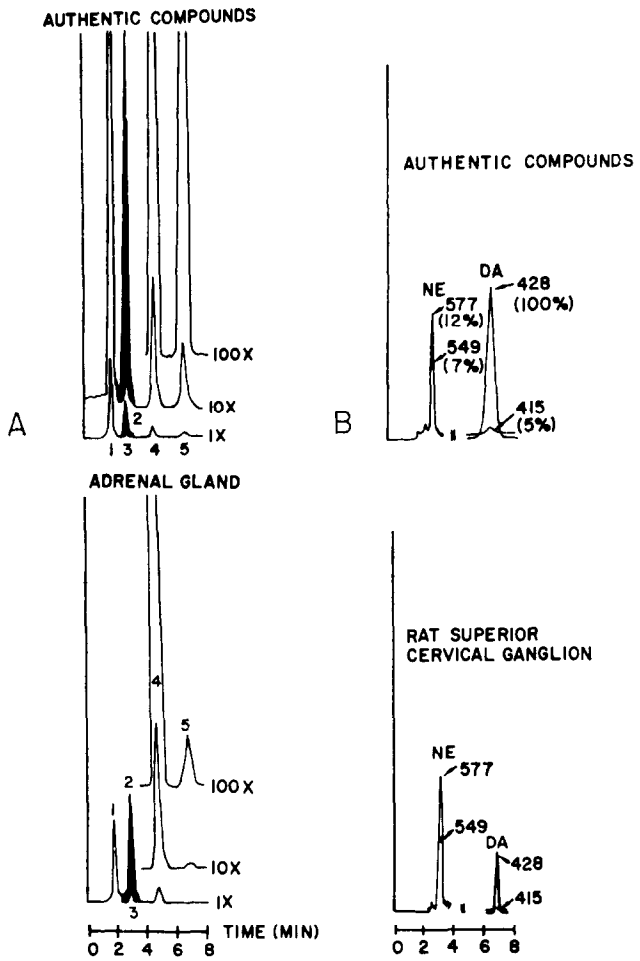

FIG. 2A.—Simultaneous analyses of norepinephrine (NE, 3); epinephrine (E, 2), and dopamine (DA, 5). Internal standards 1 α-methylnorepinephrine and 4, α-methyldopamine. For fragments recorded, see Fig. 1 or Table 1. Top record (A) authentic standards E 30 pm; NE 21 pm; DA, 47 pm. Bottom record (A) adrenal gland extract 1 adrenal gland is homogenised in 500 μl. 1 MFA (50 mM ascorbate) and 1 μl sampled for analysis E, 99·2 pm; NE, 50·6 pm; DA 3·32 pm measured. Concentration (nmoles/adrenal pair) E, 81; NE, 53; and DA, 3·4.

FIG. 2B.—Multiple ion detection. Number indicates m/e recorded. Numbers in parentheses indicate relative intensity of each fragment. Top record (B), authentic compounds. Bottom record (B) rat superior cervical ganglion extract. The ratio at m/e 577 and 549 for NE = 1·7 and the ratio at m/e 425 and 415 for DA = 20. The agreement in these ratios to those obtained from authentic compound substantiates the specificity of the measurement.

It is obvious from the GC retention times listed, that NE and E are not chromatographically resolved. It is, however, possible to resolve these compounds mass spectrometrically. NE has its base peak at m/e 176; E does not have any fragment at this m/e. The base peak for E is at m/e 190, and NE lacks any fragment at this m/e. Thus, NE is measured by recording the fragment at m/e 176, and E the fragment at 190. This can be done in a single analysis by alternating the accelerating voltage (V).

The m/e recorded by the MS is equal to:

$$\frac{H^2 r^2}{2V}$$

H^2 is the strength of the magnetic field; r^2 is the radius of curvature of the path of the ions which is a constant; and V is the accelerating voltage. To measure NE (m/e 176) the magnetic field is set to bring in focus m/e 176, r is constant and the accelerating voltage is V initial. By decreasing V, the m/e focussed would be increased (in this case to m/e 190 for E). At the retention time for NE and E one merely alternates between the two voltage settings and obtains the ion density of these fragments (Fig. 2A).

In experiments presently going on in our laboratory, we are investigating the neurotransmitter content of the cellular population of the superior cervical ganglion (SCG) of the rat. The neurons of interest are the postganglionic cell bodies, and the small intensity fluorescent cells (SIF cells). It is believed that a catecholamine released by the SIF cells modulates synaptic transmission in this ganglion (COSTA *et al.*,1961; ECCLES and LIBET, 1961). More current experiments have shown that the local application of DA hyperpolarises the membrane of ganglion cells (LIBET and TOSAKA, 1969, 1970) and in this manner may alter the responsiveness of these cells to acetylcholine released from the presynaptic elements. Consistent with the view that DA may be the neurotransmitter in the SIF cells is the histofluorometric work of BJORKLUND *et al.* (1970), indicating a high concentration of DA in sympathetic ganglion cells.

Recent histochemical experiments by ERÄNKÖ and ERÄNKÖ (1971) have indicated that the treatment of newborn rats with guanethidene results in the loss of all fluorescence from the postganglionic cell bodies, without altering the fluorescence of the SIF cells and that these SFI cells contain DA. We have reproduced these experiments in an attempt to identify by GC-MS the neurotransmitter remaining in the SIF cells. GC-MS analysis of SCG ganglion from newborn rats treated with guanethidine does not substantiate the histochemical results (Table 2). There is still at least 50 per cent of the NE remaining, and the decrease in DA is not statistically significant; this latter point does not, however, refute the theory that DA is contained in the SIF cells. As a control, histochemistry was done on the contralateral SCG of the normal and guanethidine treated rats and the results were the same as those described by ERÄNKÖ and ERÄNKÖ (1971).

To be sure of the specificity of the GC-MS measurement, multiple ion detection (MID) was done on the ganglion extract. MID takes advantage of the fact that the fragmentation pattern of any one compound is completely reproducible in terms of the

TABLE 2. EFFECT OF POSTNATAL GUANETHIDINE TREATMENT ON THE RAT SUPERIOR CERVICAL GANGLION CATHECHOLAMINE CONTENT

	Norepinephrine (pmoles/mg protein)	Dopamine
Control	746 ± 28	275 ± 57
Guanethidine	473 ± 42*	193 ± 84

New born animals were treated days 1–8 with guanethidine (20 mg/kg i.p.). Animals were sacrificed at 90 days of age. Number of animals in each group is 6. Value is the mean ± S.E.
* $P < 0{\cdot}005$.

relative intensity of each of the fragments to each other. MID varification is done by simultaneously monitoring two characteristic fragments and comparing the relative intensities of these fragments to the fragment ratios obtained from authentic compounds. Only when the ratio values are in agreement is identification considered true. MID analysis for SCG extracted catecholamine showed the NE ratio to be 1·7 and DA 20 (Fig. 2B) thereby giving an extremely high degree of specificity to the measurement.

The most direct approach for determination of the neurotransmitter content of the SIF cells would be to dissect out the SIF cells and measure the content directly. Although we believe we have the sensitivity to do this measurement, we cannot as yet dissect pure SIF cells from the ganglion. The identification of the catecholamines stored in SIF cells was, therefore, approached indirectly by making the assumption that the SIF cells contain only DA and no NE. If this is true, the ratio of DA/NE in total ganglia should be greater than the DA/NE ratio in the postganglionic cell bodies. By cutting serial 10 μm transverse sections through the ganglion, it is possible by fluorescence microscopy to find many areas where three or more successive sections contain only postganglionic cells. In order to measure the NE and DA in sections free of SIF cells, several (10 μm) sections were made and alternate sections taken for fluorescence microscopy or for possible GC-MS analysis. When two fluorescent sections were found to be free of SIF cells, then the middle section saved for GC-MS analysis was taken and the NE and DA content determined (Table 3). The average DA as a percentage of NE in ganglion sections without SIF cells is 23 per cent as compared to 37 per cent in the whole ganglion. It would appear that 41 per cent or 112 pmoles/mg protein DA is in excess and is most probably of SIF cell origin. This calculation is tested against the results with guanethidine treated animals (Table 3) and appears to hold.

TABLE 3. INDIRECT CALCULATION OF THE DOPAMINE CONCENTRATION IN SMALL INTENSELY FLUORESCENT CELLS (SIF CELLS) OF THE RAT SUPERIOR CERVICAL GANGLION

10 μm sections without SIF cells	NE (picomole/sample)	DA (picomole/sample)	$\frac{DA}{NE} \times 100$
1	1·56	0·399	25
2	2·02	0·376	19
3	0·816	0·212	26

A = Average DA as percentage NE in ganglion sections not containing SIF cells $= 23\%$

B = Average DA as percentage NE in whole ganglion with SIF cells $= 37\%$

A/B—Non SIF cell DA $= 62\%$

SIF cell DA $= 38\%$—pmoles/mg protein

Test of hypothesis:

Consider non SIF cell DA $= 62\%$

With guanethidine, NE decrease $= \underline{37\% \times}$

Then guanethidine DA should $= 23\%$

Control DA-23% = 211 pmoles/mg protein

Measured DA in guanethidine treatment = 193 pmoles/mg protein.

Alternate sections were taken for fluorescence microscopy and GC-MS analysis. Sections or parts of sections without SIF cells were pooled and analysed for amine content. No more than three 10 μm sections were analysed per sample. See text for complete discussion.

In summary we have described a method for the simultaneous measurement of NE, E and DA by gas chromatography-mass spectrometry. The sensitivity ($> 10^{-15}$ mole) and the specificity is extremely high. Since the time required to run each analysis is 5–6 min, it is extremely easy to analyse 50 samples a day, thereby generating 150 values if all three catecholamines are being measured. Using this technique, the catecholamine content of the postganglionic elements of the SCG of the rat has been measured. In comparing these values to the values obtained from whole ganglia, the DA content is apparently excessive and possibly of SIF cell origin. This conclusion is tentative and must be substantiated by direct measurement of the catecholamines in the SIF cells.

REFERENCES

BJORKLUND A., CEGVELL L., FALCK B., RITZEN M. and ROSENGREEN E. (1970) *Acta Physiol. Scand.* **78,** 334–338.
CATTABENI F., KOSLOW S. H. and COSTA E. (1972) *Science* **178,** 166–168.
COSTA E., REVZIN A. M., KUNTZMAN R., SPECTER S. and BRODIE B. B. (1961) *Science* **133,** 1822–1823.
ECCLES R. M. and LIBET B. (1961) *J. of Physiol.* **157,** 484–503.
ERÄNKÖ L. and ERÄNKÖ O. (1971) *Acta Pharmacologica (Kbh)* **30,** 403–416.
KOSLOW S. H., CATTABENI F. and COSTA E. (1972) *Science* **176,** 177–180.
LIBET B. and TOSAKA T. (1969) *J. Neurophysiol.* **321,** 43–50.
LIBET B. and TOSAKA T. (1970) *Proc. Natn. Acad. Sci.* **67,** 667–673.