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ANALYSIS OF BIOGENIC AMINES AND THEIR METABOLITES BY GAS CHROMATOGRAPHY–CHEMICAL IONIZATION MASS SPECTROMETRY

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SUMMARY

A gas chromatograph was connected to a sector-type mass spectrometer with a chemical ionization source and the performance of this combined system was studied in the analysis of biogenic amines. The system ensured a resolution of peaks as high as that of a combined gas chromatograph–electron impact mass spectrometer system. Chemical ionization mass spectrometry ensured a sensitivity more than ten times higher than that of electron impact mass spectrometry; it detected 1 pg of norepinephrine.

It was found that the ionization source temperature is an important factor in chemical ionization mass spectrometry. Suitable selection of the temperature is essential for obtaining good mass spectra.

When ammonia was used as reagent gas and the ionization source temperature was 240°, all of the fourteen biogenic amines and their metabolites gave $[M + NH_4]^+$ ions as the base peaks. Chemical ionization mass fragmentography of all of these substances was made possible by using isobutane as reagent gas.

INTRODUCTION

Biogenic amines have attracted wide attention on account of their physiological activity and many reports have appeared in the literature[1,2]. As the biogenic amines are different from each other not only in their physiological activities but also in their metabolic rates, it is necessary to carry out pharmacodynamic studies on biogenic amines, including turnover kinetics of their metabolites, in order to clarify the relationship between their structures and their physiological activity. Cerebrospinal fluids, which have recently come to be used as a material that exists close to a target organ such as the brain, contain many kinds of endogenous substances, but only trace

amounts of biogenic amines. Therefore, a highly specific method of trace analysis is required for the above purposes.

There are some reports on qualitative and quantitative analyses of these endogenous substances contained in the cerebrospinal fluids of neurological or schizophrenic patients[3].

In our previous work[4], we referred to a profile analysis of pentafluoropropionyl (PFP) derivatives of these substances, by means of a gas chromatograph and an electron impact (EI) mass spectrometer.

Chemical ionization (CI) mass spectrometry, developed by Munson and Field[5–7], has made remarkable advances because of the simplicity of its spectra and the appearance of molecular ion clusters ensures a high reliability in the determination of molecular weights, and it also provides information useful for the elucidation of structures by using various reagent gases[8]. Another advantage of CI mass spectrometry is its high sensitivity. Horning *et al.*[9] developed the important atmospheric pressure ionization mass spectrometer, which ensures an extremely high sensitivity.

In biochemical and clinical pharmacology, as the components in samples have similar structures in many cases, it is necessary to separate them before mass spectrometry. For these reasons, it has been an urgent requirement to develop a gas chromatograph–CI mass spectrometer.

Most of the combined systems so far reported involve the use of a mass spectrometer of the mass filter type[10] (quadrupole type), and there are many reports on their application[11,12]. Milne *et al.*[13] reported the application of the sector-type CI mass spectrometer using direct sample inlet CI mass spectrometry, but there have been no reports of a combined gas chromatograph–CI mass spectrometer.

This paper describes our experiments with the Shimadzu LKB 9000 instrument with a CI source attached. The purpose of our experiments was the qualitative and quantitative analyses of biogenic amines contained in cerebrospinal fluids by obtaining information on molecular weights of samples and increasing the sensitivity of detection.

EXPERIMENTAL

Materials

Dopamine (DA), norepinephrine (NE), epinephrine (E), normetanephrine (NM), 3-methoxytyramine (3-MTy), 6-hydroxydopamine (6-HDA), homovanillic acid (HVA), vanillylmandelic acid (VMA), 5-hydroxytryptamine (5-HT), indole-3-acetic acid (IAA), 5-hydroxyindole-3-acetic acid (5-HIAA), tryptamine (T), 5-methoxytryptamine (5-MT) and N-methyltryptamine (NMT) were purchased from Tokyo Kasei Co., Tokyo, Japan. Pentafluoropropionic acid (PFPA) was purchased in 200-ml bottles from Pierce, Rockford, Ill., U.S.A. Pentafluoropropionyl-1,2,4-triazole (PFPT) was synthesized by the method reported in our previous paper[4].

Equipment and operating conditions

Gas chromatograph–CI mass spectrometer: Shimadzu LKB 9000 (Shimadzu Seisakusho Ltd., Kyoto, Japan), with an improved version of a chemical ionization source, manufactured by Scientific Research Instruments Corp., Baltimore, Md., U.SA., attached.

Multiple ion detector (MID): Shimadzu MID-PM 9060S.
Gas chromatograph column: OV-101, 3% on Chromosorb W-HP, 100–120 mesh, 1 m long (Applied Science Labs., State College, Pa., U.S.A.).
Column temperature: 180° or 200°.
Flash heater temperature: 250°.
Ion source temperature: 230–320°.
Carrier gas helium flow-rate: 30 ml/min.
Ionization potential: 500 eV.
Accelerating voltage: 3500 V.
Pressure in ionization chamber: 0.5–1 torr.
Pressure in analyzer unit: 10^{-6} torr.
Reagent gas: methane, isobutane or ammonia.

Derivatization

Biogenic amines (1–2 μg) were dissolved in 0.1 ml of PFPT and allowed to stand at room temperature for 30 min in a tightly stoppered micro-tube. Thus all the biogenic amines were PFP derivatized.

Amounts of 1–2 μg each of the acids (HVA, VMA, IAA and 5-HIAA) were dissolved in 1 ml of a 1 *N* solution of PFPA in methanol. The mixture was heated at 60° for 1 h so as to esterify the carboxylic acid groups. The excess of PFPA–methanol solution was removed under reduced pressure, then 0.1 ml of PFPT was added and derivatization was performed in the same way as for amines. Thus the acids were derivatized into methyl ester PFP derivatives and the structures were confirmed by using the EI mass spectrometer.

RESULTS AND DISCUSSION

Construction and performance of combined gas chromatograph–chemical ionization mass spectrometer system

The systems for introducing effluent from the gas chromatograph into a sector-type mass spectrometer can be classified into three types: splitter system, direct connection system and separator system.

The splitter system has the disadvantage that a high sensitivity of detection cannot be expected because the effluent from the gas chromatograph is split before it is introduced into the ionization chamber. The direct connection system has the disadvantage that it requires a higher vacuum in the ionization chamber than in the case of the mass filter system, in order to remove completely carrier gas which might have an unfavourable effect upon the ionization process.

We therefore employed the separator system, which has the advantages that it can fully utilize the features of the Shimadzu LKB 9000 instrument and that it is easy to change the reagent gases. The chemical ionization source was modified as shown in Fig. 1[14].

One of the most important points when combining a gas chromatograph and a chemical ionization mass spectrometer is to prevent components of adjacent peaks from mixing with each other while they travel, after being ionized, from the ionization chamber into the high-vacuum chamber. As the ionization chamber is maintained at a vacuum of about 1.0 torr, while the high-vacuum chamber is kept at 10^{-6} torr,

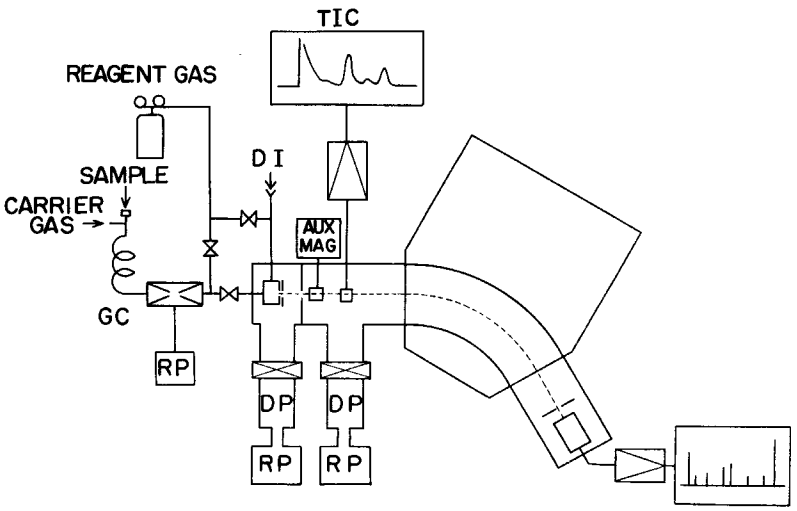


Fig. 1. Schematic diagram of combined gas chromatograph–chemical ionization mass spectrometer system. As the carrier gas ions are deflected by the AUX MAG, only the ion current of the sample is recorded by TIC. DI = direct inlet; RP = rotary pump; DP = diffusion pump; TIC = total ion current recorder; AUX MAG = auxiliary magnet.

and the two chamber are divided by a wall with a small hole, closely eluted components are likely to become mixed as a result of diffusion and convection. In order to measure the degree of mixing in our equipment, we analyzed a mixture of NE and E, which have approximately the same retention times and which give different CI mass spectra.

As shown in the chromatogram obtained by the total ion current recorder (Fig. 2), when methane or isobutane is used as the reagent gas, the two compounds are separated with a resolution as good as that obtained with a gas chromatograph – EI mass spectrometer.

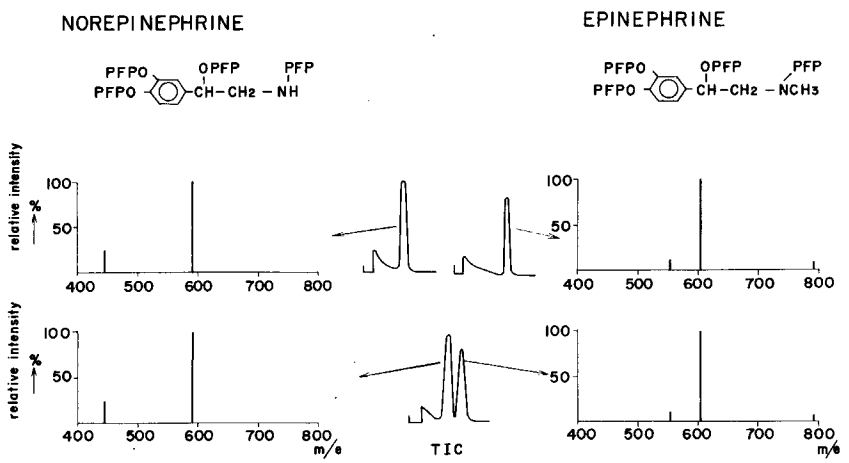


Fig. 2. Separation of norepinephrine and epinephrine by combined gas chromatography–CI mass spectrometry. Above: the two compounds were separately introduced. Below: the two compounds were introduced as a mixture. Reagent gas: methone or isobutane.

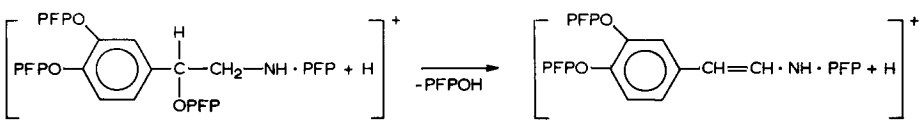
Derivatization

The fluorinated acylating reagents for biogenic amines are used in the form of their anhydrides[15] for catecholamines and their imidazoles[16] for indoleamines. However, there are no reagents available that can derivatize both of them, so we synthesized the reagent pentafluoropropionyl-1,2,4-triazole for this purpose. This reagent can completely acylate the active hydrogen atoms of catecholamines but does not acylate the NH group in indolic compounds such as 5-HT, thus providing peaks of high reliability and high stability[4].

Chemical ionization with various reagent gases

It is reported[15] that, in the EI mass spectrometry of catecholamines, molecular ions are hardly produced, only a few per cent at the maximum, because the fragmentation proceeds mainly as α- or β-cleavage.

We studied the fragmentation of the PFP derivatives of these substances using methane, isobutane or ammonia as the reagent gas. When methane or isobutane was used, $[M + H]^+$ ions were recorded as the base peaks of DA and 6-HDA. Isobutane gave even simpler spectra than methane (Fig. 3). In analysis of NE, however, contrary to the report of Milne *et al.*[13], elimination of PFPOH from the derivatives was observed according to the equation



The fragment ion formed in this fragmentation is recorded as the base peak, and the CI spectra obtained were similar to those obtained by EI mass spectrometry. Therefore, NE and 6-HDA, which have almost identical retention times under these gas chromatographic conditions and give similar EI mass spectra, can be easily differentiated from each other by CI mass fragmentography using isobutane as the reagent gas (Fig. 5). A detailed explanation is given later.

Indoleamines such as 5-HT and 5-MT gave typical CI spectra when isobutane was used as the reagent gas, MH^+ being recorded as the base peak. The metabolites, such as 5-HIAA and HVA, also gave spectra with MH^+ as the base peaks.

NE, which did not give the quasi-molecular ion when isobutane was used as the reagent gas, gave an $[M + NH_4]^+$ fragment ion when ammonia was used as the reagent gas. The metabolites such as HVA, VMA, IAA and 5-HIAA also gave $[M + NH_4]^+$ fragments when ammonia was used as the reagent gas.

Influence of ionization source temperature on CI spectra

Field[17] reported the influence of the ionization source temperature on CI spectra. We also studied this influence and obtained the following results.

When NE was measured with an ionization source temperature varying from 230 to 320°, using ammonia as a reagent gas, a remarkable change in the CI spectra was observed, as shown in Table I. All of the substances except IAA and 5-MT gave CI spectra with only $[M + NH_4]^+$ peak when the ionization source temperature was

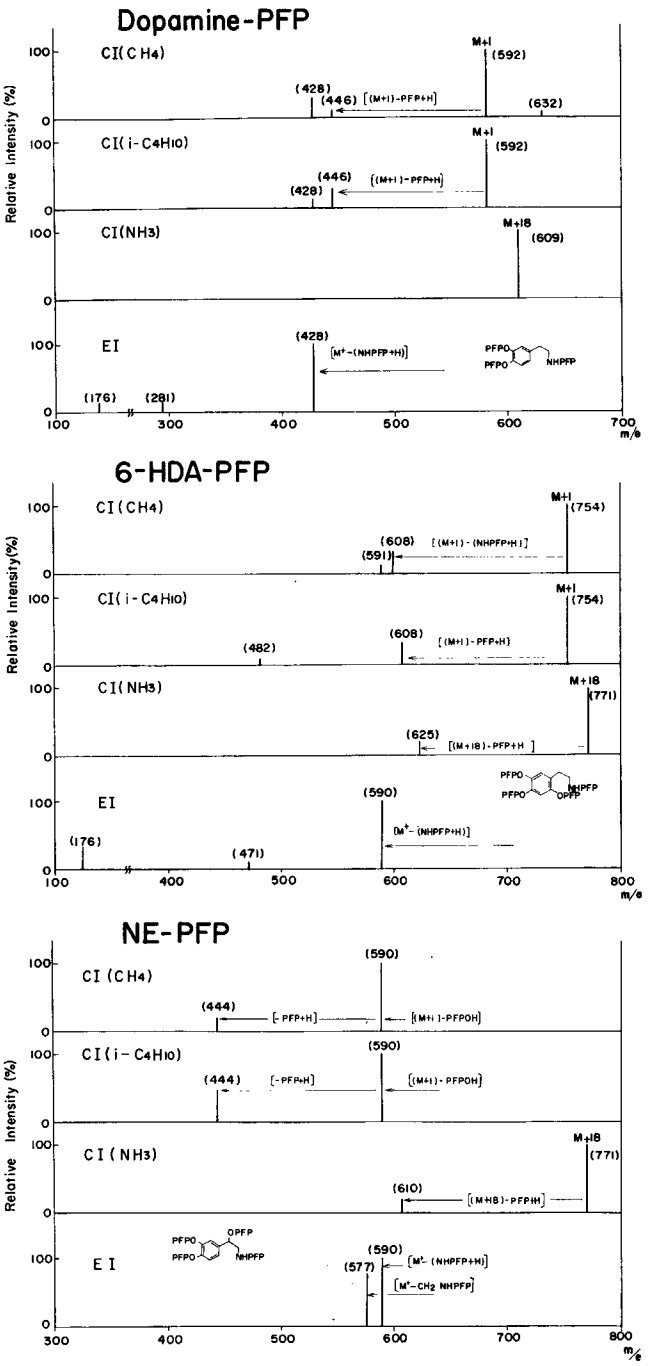


Fig. 3. Analysis of dopamine, 6-hydroxydopamine and norepinephrine by gas chromatography–CI mass spectrometry and gas chromatography–EI mass spectrometry. In gas chromatography–CI mass spectrometry, methane, isobutane or ammonia was used as the reagent gas. The temperatures of column, flash heater and ionization source were 180°, 250° and 270°, respectively.

270°. IAA and 5-MT did not give $[M + NH_4]^+$ as the base peak when the ionization source temperature was 270°, but when the temperature was lowered to 240°, all of the fourteen biogenic amines gave $[M + NH_4]^+$ ions as base peaks (Table II). With an increase in the ionization source temperature, 6-HDA gave more fragment ions in the low mass region and a smaller quasi-molecular ion peak (Table I). This temperature dependence was especially significant with NE: at an ionization source temperature of 320°, the relative intensity of the quasi-molecular ion decreased to one quarter and the amount of total ions also decreased considerably.

When these results are taken into consideration, it may be possible to differentiate between NE and 6-HDA, which are difficult to separate by conventional methods, within the mass range of the MID of a sector-type mass spectrometer.

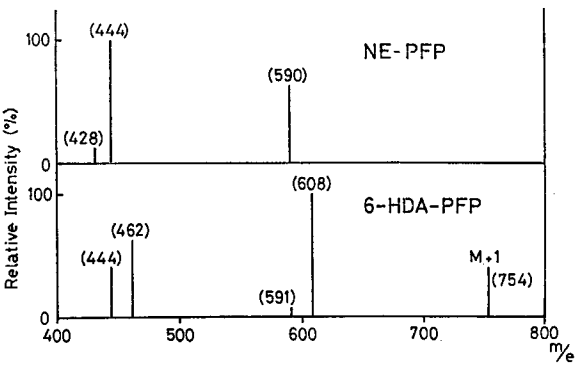


Fig. 4. CI mass spectrometric spectra of norepinephrine and 6-hydroxydopamine with ionization source at 310°. Reagent gas: isobutane.

Fig. 4 shows the mass spectra of NE and 6-HDA when isobutane was used as the reagent gas and the ionization source temperature was 310°. Fig. 5 shows the results of mass fragmentography focused on *m/e* 590 for NE and on *m/e* 608 for 6-HDA.

Chemical ionization mass fragmentography

Although mass chromatography is suitable for the profile analysis of these fourteen biogenic amines, we adopted mass fragmentography because it has a higher sensitivity.

Use of ammonia as the reagent gas is advantageous in that it provides a quasi-molecular ion as the base peak, but it cannot differentiate NE from 6-HDA. We therefore used isobutane in the mass fragmentography of these compounds, and the results are shown in Fig. 6.

Sensitivity of detection of chemical ionization mass fragmentography

It is well known that the sensitivity of mass fragmentography differs considerably with the types of samples used. We reported[4] that the limit of detection of EI mass

TABLE I

RELATIVE INTENSITIES (%) OF FRAGMENT IONS OF NOREPINEPHRINE AND 6-HYDROXYDOPAMINE FOR VARIOUS IONIZATION SOURCE TEMPERATURES

Reagent gas: ammonia.

Temp. of ion source (°C)	*Fragment ion of norepinephrine (m/e)*										*Fragment ion of 6-hydroxydopamine (m/e)*										
	771	*720*	*610*	*590*	*452*	*444*	*429*	*384*	*368*	*295*	*771*	*720*	*625*	*534*	*478*	*444*	*384*	*368*	*295*	*237*	*191*
230	100	—	37.7	—	—	—	—	—	—	—	100	6.8	50.6	6.8	6.8	—	—	—	—	—	—
270	100	10.6	38.6	—	—	20.0	—	—	—	—	100	5.6	37.7	—	—	—	—	—	—	—	—
300	62.5	—	50.0	42.8	35.7	100	75.0	39.2	75.0	32.1	100	15.3	82.0	—	—	43.5	15.3	64.1	33.3	—	—
320	25.0	—	—	36.3	52.2	95.4	79.5	63.6	100	81.8	21.6	—	16.2	—	—	54.0	35.1	100	62.1	24.3	32.4

TABLE II

FRAGMENT IONS OF BIOGENIC AMINES AND THEIR METABOLITES WHEN AMMONIA WAS USED AS THE REAGENT GAS

Ionization source temperature: 270°. The values are relative intensities (%).

PFP derivative	*Fragment ions*				
	$[M + NH_4]^+$	*$[M + H]^+$*	*$[(M + 18)\text{-}NHPFP + H]^+$*	*$[(M + 18)\text{-}PFP + H]^+$*	*$[(M + 18)\text{-}PFPOH + H]^+$*
DA	100	—	—	—	—
NE	100	—	20	—	—
E	100	—	—	—	—
NM	100	—	—	—	—
3-MTy	100	—	—	—	—
6-HDA	100	—	—	21	—
HVA	100	—	—	—	—
VMA	100	—	—	—	93
5-HT	100	—	—	—	—
IAA*	100	21	—	—	—
5-HIAA	100	—	—	—	—
T	100	28	—	—	—
5-MT*	100	—	37	—	—
NMT	100	19	—	—	—

* Ionization source temperature is 240°.

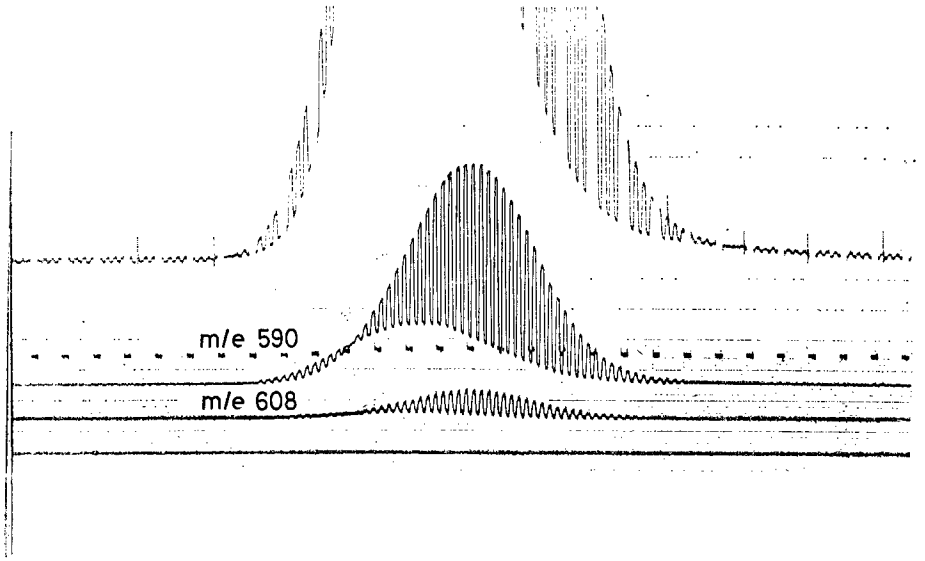
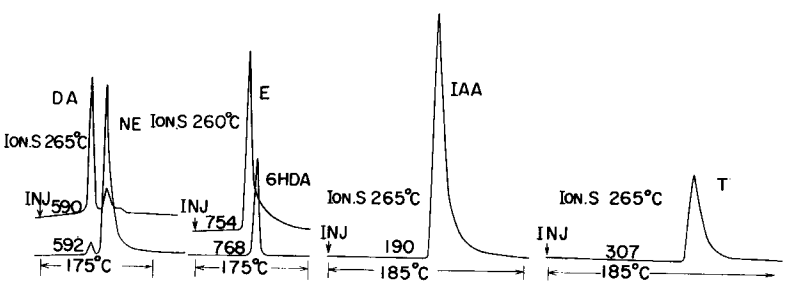


Fig. 5. Differentiation of norepinephrine and 6-hydroxydopamine by mass fragmentography. An accelerating voltage alternator was used for focusing on *m/e* 590 for NE and on *m/e* 608 for 6-HDA.



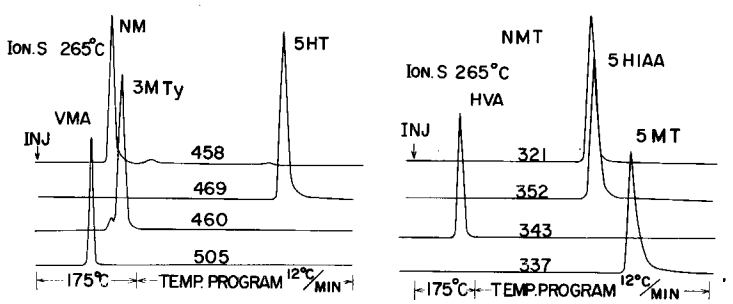


Fig. 6. Mass fragmentograms of biogenic amines and their metabolites. Reagent gas: isobutane. Amount of sample injected: 1 ng. ION. S = ionization source temperature; INJ = injection.

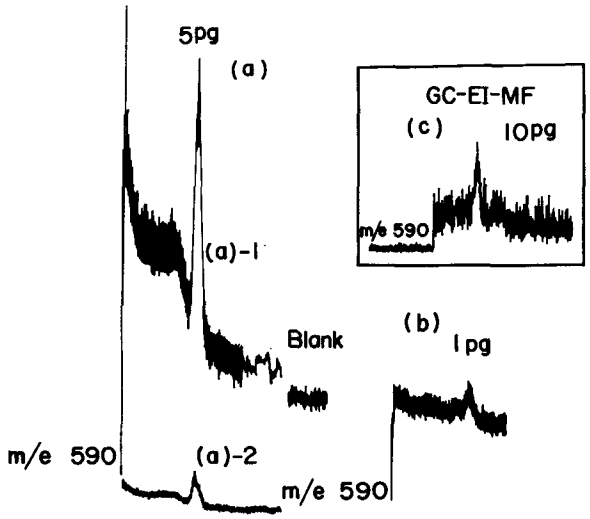


Fig. 7. Mass fragmentograms of norepinephrine by CI and EI mass spectrometry. CI mass spectrometry: isobutane as reagent gas; focusing on *m/e* 590. EI mass spectrometry: focusing on *m/e* 590. (a) Sample size, 5 pg; gain, 10; slit width, 0.3 mm, 0.3 mm. (a-2) The range was set to one tenth of a-1, so the amount corresponds to 500 fg. Blank shows the chromatogram at *m/e* 590 for 1 μl acetone. (b) Sample size, 1 pg; gain, 10; slit width, 0.3 mm, 0.3 mm. (c) Sample size, 10 pg; gain, 8; slit width, 0.4 mm, 0.5 mm; focusing, *m/e* 590.

fragmentography for NE was 10 pg. Kiryushkin and Fales[18] reported that the sensitivity increased when CI was used.

The sensitivities of EI and CI mass fragmentography were measured at *m/e* 590, using isobutane as the reagent gas. It is clear from Fig. 7 that the adoption of CI instead of EI enhanced the sensitivity by a factor of about 10.

In order to investigate whether or not quantification is possible at such an

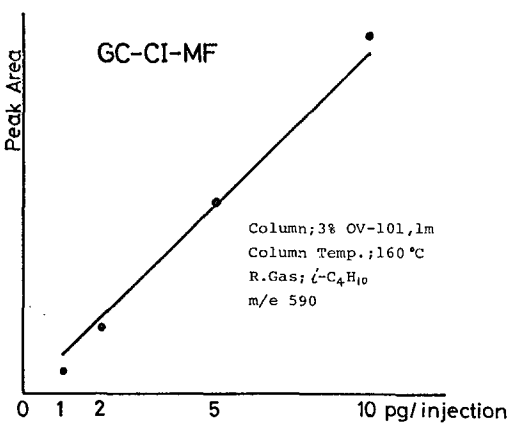


Fig. 8. Absolute calibration curve for CI mass fragmentography of norepinephrine: 1, 2, 5 and 10 pg of norepinephrine were injected and mass fragmentography was performed at *m/e* 590. The peak areas were plotted against the sample sizes.

ultra-micro level, we prepared a calibration curve by adding known amounts of samples without internal standard (absolute calibration method). The calibration curve obtained has excellent linearity, as shown in Fig. 8.

CONCLUSION

It has been shown experimentally, by using a chemical ionization source in mass fragmentography, that CI mass spectrometry ensures a higher sensitivity than EI mass spectrometry and that the former enables qualitative and quantitative analyses to be carried out at the picogram level. This technique leads to further simplicity in the quantitative analysis of biogenic amines and their metabolites contained in cerebrospinal fluids. The results show that combined gas chromatography–CI mass spectrometry will be an effective method for use in clinical chemistry, research into biological substances and the study of metabolism.

The simplicity of fragmentation and the formation of quasi-molecular ions, which are the advantages of CI mass spectrometry, will be a disadvantage in the differentiation of isomers. However, when gas chromatography–CI mass spectrometry is used, this disadvantage can be obviated by separating isomers before they are introduced into the chemical ionization chamber. Moreover, differentiation of isomers that cannot be distinguished by EI mass spectrometry has become possible by suitable selection of the reagent gas.

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