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RAPID IDENTIFICATION OF LOW MOLECULAR WEIGHT COMPOUNDS IN EXTRACTS OF BIOLOGICAL MATERIAL

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SUMMARY

The problem of obtaining rapid information about the elementary composition of low molecular weight compounds in submicrogram quantities in complex mixtures without prior isolation has been simplified by the development of a new technique, involving the use of a combination of gas chromatography and mass spectrometry (GC–MS). A peak matching accessory allows the subsequent matching of the characteristics of the molecular ion of an unknown compound with that of a reference substance having a known mass adjacent to that of the unknown. The precise molecular weight of the unknown molecular ion can then be calculated with an error of 5–10 p.p.m. This calculated value is then compared with those recorded in appropriate reference manuals in order to obtain the empirical formula of the unknown compound. The mass spectrum of the reference compound corresponding to the most appropriate empirical formula is compared to the spectrum of the unknown to verify its identity. This technique led to the detection and identification of dimethylaminoethanol and piperidine in rat brain.

INTRODUCTION

The identification of unknown compounds in the effluent of a gas chromatograph (GC) has been partially simplified by the use of a system whereby the column effluent passes directly into a mass spectrometer (MS)[1]. However, the analysis of low resolution mass spectra of unknown compounds generally requires a high index of suspicion as to their identity, so that comparisons can be made with the fragmentation patterns of appropriate reference substances. Computer analysis does facilitate the process of identification but is not yet generally available. This paper reports the use of a new GC–MS technique which has been developed as an aid to the rapid identification of unknown volatile substances in rat brain extracts. During long term biological studies of acetylcholine using various techniques we became interested in other cerebral amines. In general, the methods of HOLMSTEDT AND LUNDGREN[2] and HANIN AND

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JENDEN[3] have been used for the brain preparation, extraction and gas chromatographic studies.

MATERIALS AND METHODS

Brain preparation

The brains of albino, male, 200–225-g Sprague–Dawley rats were prepared and extracted according to the method described for acetylcholine by HANIN AND JENDEN[3]. In the latter technique, acetylcholine is demethylated and extracted into chloroform by raising the pH of the water phase to about 9. The more polar amines remaining in the water phase were *not* studied. In our current experiments the highly polar amines were extracted by the addition of 0.1 ml of 13.5 *M* KOH and 0.05 ml chloroform to a 0.1 ml aliquot of the water phase. The combined solution was mixed vigorously for 2 min and centrifuged at 2000 × *g* for 2 min. A 1-μl aliquot of the chloroform phase was used for the gas chromatographic studies.

Gas chromatography

A Varian model 1200 gas chromatograph equipped with a hydrogen flame ionisation detector was used. The silanised glass column measured 1.5 mm (I.D.) by 1.6 m. Unsilanised, acid- and ammonia-washed Chromosorb W (80–100 mesh) was coated with 4% Amine 220 (1-hydroxy-2-heptodecenyl-imidazoline) and 6% THEED (tetrahydroxyethylenediamine). Injector and detector temperatures were maintained at 150° and 200°, respectively. Flow rates were about 20 ml/min for nitrogen (carrier gas), 200 ml/min for oxygen and 25 ml/min for hydrogen. The detector response was recorded on a Servogor model RE 512 recorder.

Gas chromatography–mass spectrometry

The combined gas chromatograph–mass spectrometer, LKB 9000 (LKB-Produkter AB, Fack, 161 51 Bromma 1, Sweden) was used to scan the mass spectra of the components as they were eluted from the GC column (similar to that described above).

An accessory to this GC–MS system, the LKB 9020 oscilloscopic peak matching device, was used to measure the ratio of the mass of an appropriate ion of an unknown compound to that of a reference agent or *vice versa*.

RESULTS

Gas chromatography

In the initial chromatographic determination 1 μl of the chloroform phase was injected; the column temperature was maintained at 30° until the solvent front and first compound had been completely eluted; the temperature was then increased 8°/min to 70° where it was held until the remaining compounds had been eluted (Fig. 1). The new technique involving the combined gas chromatograph–mass spectrometer–peak matcher system was then used to identify rapidly the compounds eluting as individual peaks (see below). After identification the compounds of interest were studied isothermally at a column temperature of 60° (Fig. 4).

Gas chromatography–mass spectrometry

The identity of one compound only was suspected in the gas chromatogram of the rat brain extract (Fig. 1). Synthetic dimethylaminoethanol (DMAE) has a retention time equal to that of the last peak. In order to establish its identity, a mass spectrum of that peak in the rat brain extract was recorded by means of the combined gas chromatograph–mass spectrometer, LKB 9000. This spectrum was compared to that of synthetic dimethylaminoethanol (Fig. 2). The resemblance was such that the identity could be confirmed.

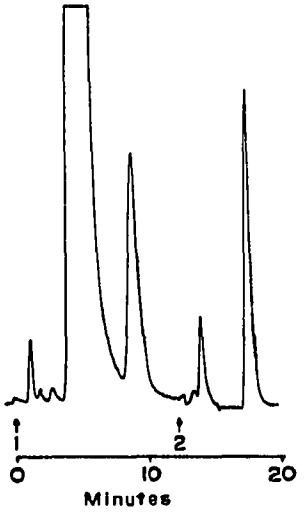

Fig. 1. GC separation of volatile compounds in a 1-μl chloroform aliquot of rat brain extract. Column temperature was kept at 30° between 1 and 2 and at 70° from 2 to the end of the trace. Temperature was raised from 30° to 70° at 2 and the baseline adjusted. First peak after the chloroform solvent front corresponds to the elution of ethanol followed by piperidine and dimethylaminoethanol.

The mass spectrum of the compound eluted just before DMAE was also recorded. The interpretation of the spectrum of this unknown compound was difficult due to interference by unresolved components. Serial scans were recorded, as the compound was eluted, at approximately the same interval to make it possible to distinguish between ions belonging to the interfering compound(s). Instead of scanning successively we could have taken advantage of the selectivity of the mass fragmentographic technique[4] and thus achieve the same picture of the variations of the intensities of the m/e values as shown in Fig. 3.

Twelve successive scans in the mass range 65 to 130 were recorded during the eluting time of the compound. The intensities (in mm) of the different mass numbers were plotted against the successive numbers of scans which are analogous to the time (Fig. 3). The fragments characteristic of the compound vary in intensity while the intensities of the interfering ions are relatively constant. The highest m/e value of the ions of varying intensities, $m/e = 85$, was selected as the most probable parent ion of the unknown compound.

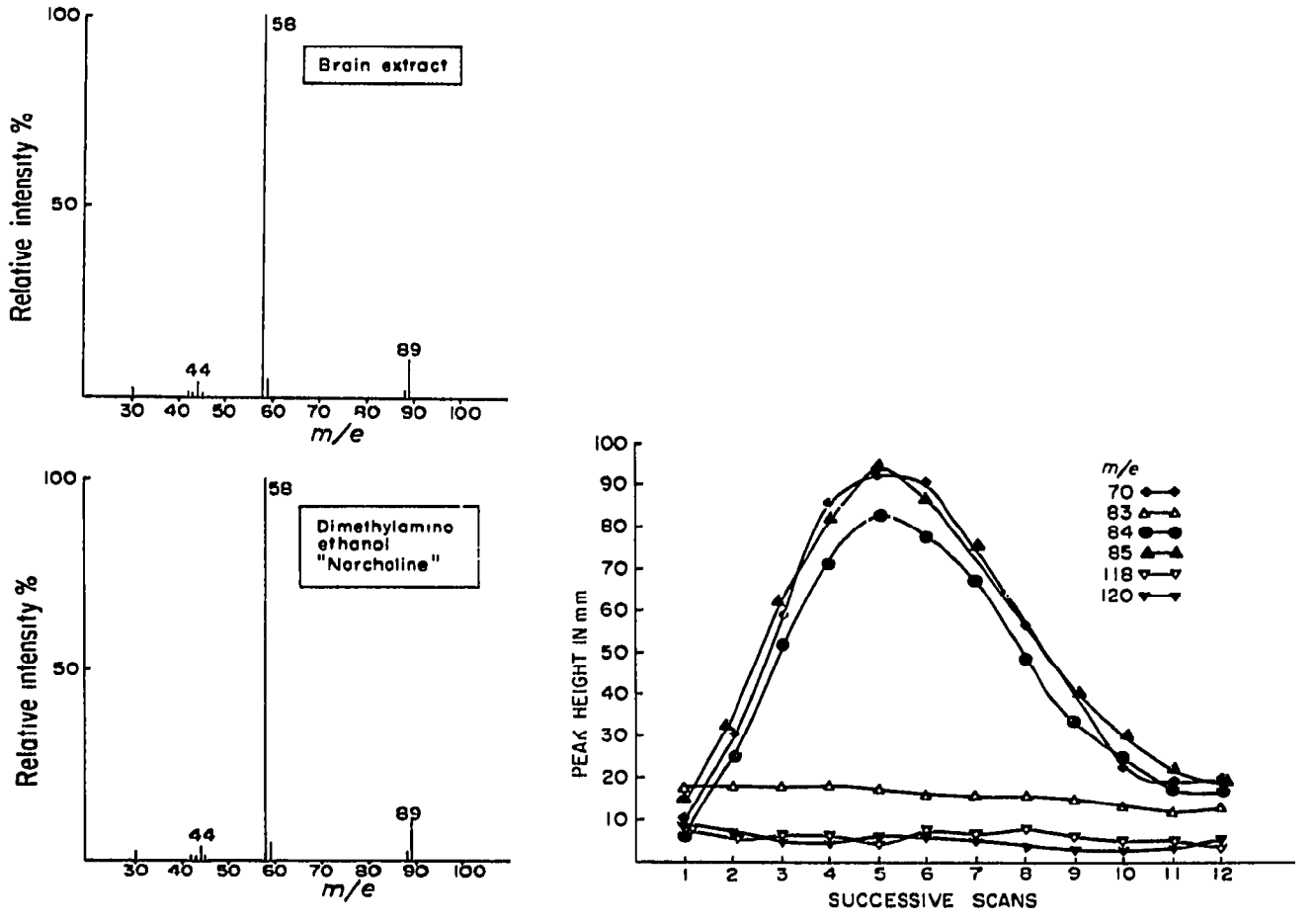

Fig 2. Upper panel mass spectrum of a compound extracted from rat brain and having the same retention time as synthetic dimethylaminoethanol. Lower panel mass spectrum of synthetic dimethylaminoethanol The mass spectra were recorded at an ionisation energy of 70 eV GC conditions as in the legend to Fig. 1, except that the temperature was maintained isothermally at 60°.

Fig. 3 Changes in the intensities of six selected masses during twelve successive scans of a compound eluting from the GC column (see text) GC and MS conditions as in the legend to Fig 2.

New peak matching technique of non-isolated compounds

The usual principles when operating with isolated compounds may be summarised as follows:

The intensity of ions of a certain mass is visualised as a Gaussian curve on the oscilloscope screen of the peak matcher. Ions of lower mass, *e.g.*, the parent ions of the unknown compound, are brought into focus by a manual change of the magnetic field. Ions of higher mass, *e.g.*, those of the reference compound, are then brought into focus by keeping the magnetic field unchanged while the accelerating voltage is decreased by manual adjustment of a high precision decade resistor, calibrated in mass ratio 1.0^{++++++}. The second to the sixth decimals are adjustable. A relay is then set to switch the accelerating voltage between full and reduced, low and high mass (unknown and reference) which makes it possible to watch both masses on the oscilloscopic screen and match them. A six-decimal value for the weight (a.m.u.) of

the unknown compound can then be calculated and an empirical formula deduced. (The last two or three decimals are of little significance.)

This standard peak matching technique was modified to allow peak matching during the elution of an unknown compound from the column. As the time to match the peaks is short and equals the elution time, the precision decade must be set to a pre-selected value of the expected ratio. A list of organic compounds[5] having molecular weights of 85 was inspected and 2-pyrrolidone, C_4H_7NO, with a molecular weight of 85.052761 selected as possible. This value was divided into 86.109545, the molecular weight of the selected reference, *n*-hexane, C_6H_{14}. M^+ hexane/M^+ pyrrolidone = 86.109545/85.052761 = 1.012425. This ratio was pre-set on the precision decade of the peak matcher.

If the unknown compound is pyrrolidone, then, as the compound eluted, the position of the peak appearing on the oscilloscopic screen should be superimposed on that of the reference, *i.e.* match perfectly. This was not the case—the compound was therefore not pyrrolidone. However, the unknown peak did resemble that of $(M_{\text{hexane}}{}^{-1})^+$ (C_6H_{13} = 85.101720) which also appeared on the oscilloscope screen. Reference tables[5] indicate that the organic compound with a mass closest to C_6H_{13} is piperidine, $C_5H_{11}N$ (M = 85.089145).

A new reference, dichloromethane, was selected because it does not interfere with the molecular ion of the unknown. The mass ratio was calculated M^+ piperidine/M^+ dichloromethane = 85.089145/83.953355 = 1.013529 and the value pre-set on the decade. When the peak of the unknown compound appeared on the screen it could be superimposed on that of dichloromethane—strongly suggesting that the unknown compound is piperidine. The identity was then confirmed by gas chromatography (Fig. 4) and gas chromatography–mass spectrometry.

The gas chromatographic peak appearing just after the chloroform at a column temperature of 30° was also examined as described above (Fig. 1). The mass spectrum

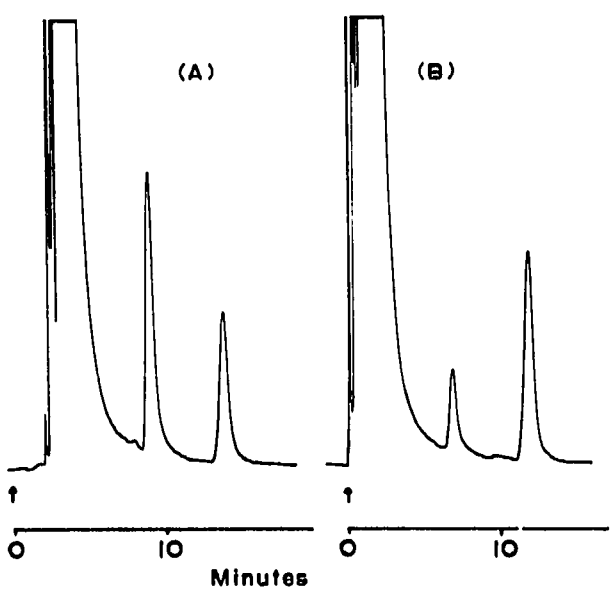

Fig. 4. (A) Gas chromatographic separation of 2 µl of a 5 m*M* solution of piperidine (first peak) and dimethylaminoethanol (second peak). Conditions are the same as in the legend to Fig 2. (B) Gas chromatogram of 1 µl rat brain extract (see text). GC conditions as in A.

of the compound eluting at this peak indicated that the highest mass numbers were 46 and 45. We selected from the reference tables an empirical formula for the compound of C_2H_7N, with a mass value of 45.057846 (m/e = 46 could then be $(M+1)^+$). This formula was selected because extraction characteristics of the unknown indicated a basic compound.

CO_2 present in the carrier gas was used as the reference. The pre-set mass ratio was $M^+ C_2H_7N/M^+ CO_2$ = 45.057846/43.989828 = 1.0242787. The choice was incorrect as the Gaussian curve of the eluting compound was not superimposed on that of the reference. The ratio was rapidly altered until the peaks approximately matched. The value of the new ratio was 1.0237. This ratio multiplied by the accurate weight of carbon dioxide gave the mass of the unknown as 45.032386. The closest value in the reference table[5] is 45.034037 with an empirical formula of C_2H_5O. Dividing 45.034037, (C_2H_5O) by 43.989828 (CO_2) gives the ratio 1.023737. This calculated ratio is in very good agreement with the measured value. We concluded that the matched ion had the composition C_2H_5O and instead of being the molecular ion it was the parent ion minus one hydrogen. The molecular ion should then be C_2H_6O, *e.g.* ethanol. This suspicion was confirmed by gas chromatography and mass spectrometry. 1% ethanol had been added to the stock solution of chloroform for stabilisation purposes.

DISCUSSION

Usually peak matching of an unknown compound against a known reference requires that the unknown be isolated in μg-quantities[6]. This isolation may be difficult and time-consuming, especially when working with biological material where the amounts available are usually small. The technique we describe makes it possible to match the peak of an unknown component in a mixture without prior isolation; the matching is done as the component is eluted from the gas chromatographic column into the mass spectrometer. If another ion is present and has almost the same mass as that of the unknown ion the single focussing instrument may lack sufficient resolving power to perform this peak matching technique.

The determination of the mass number of the molecular ion is done by scanning a mass spectrum. This information, the fragmentation pattern and the extraction characteristics of the compound are the basis for the first guess as to the identity of the unknown. The mass ratio between the unknown compound and the reference or *vice versa* is pre-set on the peak matching device. If the guess is right the two peaks representing the two compounds will be superimposed on the oscilloscopic screen. If the guess is incorrect a rapid estimation of the ratio can be made. Then a new assessment, based upon this estimation, is tested. Two or three injections of the unknown solution may be required before the accurate mass ratio is determined. The principal advantage of the technique is that it gives rapid information about the elementary composition of compounds in complex mixtures in submicrogram quantities without prior isolation. In this paper the identity of the unknown compounds was confirmed by comparing their mass spectra to synthetic dimethylaminoethanol and piperidine.

The technique of JENDEN, HANIN AND LAMB[7,8] was modified to permit the recovery of choline, dimethylaminoethanol and piperidine Simultaneous estimation of dimethylaminoethanol and dimethylaminoethyl acetate (the demethylation product of acetylcholine) is difficult with the 'Polypak 1'–phenyldiethanolamine succinate

support–stationary phase combination originally described[7] because of temperature instability and adsorption effects. Therefore, a stable and less active support, Chromosorb W, and a stationary phase mixture of 4% Amine 220 and 6% THEED was adopted which provides a very satisfactory separation of the volatile amines at low temperature (Figs. 1 and 4). The reduced bleeding greatly facilitated the mass spectrometric studies.

The choline content of rat brain has been estimated by a variety of methods[9,10]. The technique of HANIN AND JENDEN results in the hydrolysis of the dimethylaminoethyl acetate and the subsequent determination of the total dimethylaminoethanol[3]. In the present study both compounds may be determined separately so that the concentration of both may be measured.

Piperidine was first reported by HONEGGER AND HONEGGER[11] as a normal constituent of brain and has previously been found in human CSF[12] and urine[14] as well. Although its origin is not known it is thought to be a product of lysine metabolism through decarboxylation of the intermediate metabolite, pipecolic acid and/or deamination and cyclisation of cadaverine[15]. Certain investigators have suggested that piperidine may function as an endogenous synaptic regulatory substance modulating behaviour[16]. It has been shown to effect markedly the behaviour and activities of human patients and test animals[17]. Other quantitative data and the effect of psychoactive drug treatment and environmental conditions on the levels of these biogenic amines in rat brain will be discussed in future communications.

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