APPLICATIONS OF INTEGRATED GAS CHROMATOGRAPHY/MASS SPECTROMETRY IN PHARMACOLOGY AND TOXICOLOGY

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Gas chromatography is widely recognized as one of the most powerful separative techniques available, and is widely used in biomedical analysis (1, 2, 3). The very high sensitivity of the flame ionization detector and electron capture detector are essential for many applications to the measurement of drugs, metabolites, and environmental poisons that may be present in biological samples only in trace amounts. Although the electron capture detector and other specialized detectors confer a considerable relative specificity for certain types of compounds (4), they provide very little information regarding the identity and molecular structure of compounds eluting from the GC column. On the other hand, the mass spectrum of an unknown compound can give considerable information about its structure, and if compared with the spectrum of an authentic reference compound, can rigorously establish it. An additional feature of mass spectrometry of interest in this context is the small (10–100 ng) sample requirement. These features have been exploited in the biological and medical sciences and several reviews have appeared on the subject (5, 6).

A logical extension of these two techniques is an integrated instrumental system in which a gas chromatograph (GC) is coupled to a mass spectrometer (MS) utilizing both instruments to maximal advantage (7–10). Under favorable circumstances this allows every major component of a complex mixture to be identified (see, e.g. 11).

Coupling the two types of instruments presented a series of technical problems which have been reviewed by Ställberg-Stenhagen & Stenhagen (12). The most difficult problem arises from differences in operating pressures between the two systems. The MS is operated at high vacuum, so that most of the carrier gas must be removed from the GC effluent. This is achieved by a molecule separator, of which many types are now available (13–15).

The information provided by a mass spectrometer relates to the highly characteristic pattern of fragments into which a molecule splits when it is ionized in the vapor phase. Both the masses of the principal fragments and their relative abundance are characteristic of a given molecule under a constant set of conditions. These data constitute a mass spectrum, and are generally represented as a bar graph normalized so that the most abundant ion (base peak) has a relative abundance of 100% (Figure 1). The abscissa is the mass/charge ratio generally designated m/e, since the separation of ions depends on this physical property.

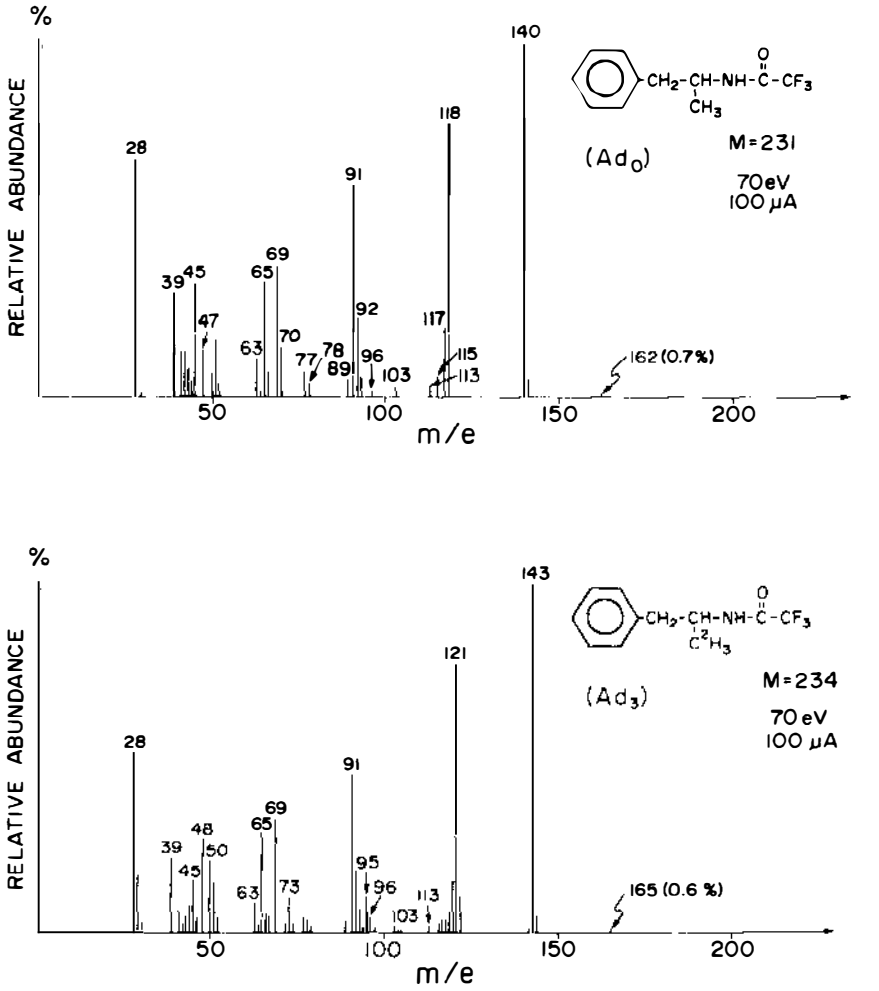

FIG. 1. Mass spectra of the trifluoroacetamides of amphetamine (Ad_0) and trideuteroamphetamine (Ad_3). Note that the lines corresponding to m/e 140 and 118 in Ad_0 are shifted to 143 and 121 respectively in the deuterium substituted compound, Ad_3. (See reference 16 for further details.)

In most cases, the charge is unity and the ratios are therefore close to integers which correspond to the sum of the atomic weights of the fragment. These integers are referred to as the "nominal mass" of the fragment, and differ from the precise masses because the masses of nuclides are not simple multiples of a fundamental unit; for example the precise mass of a $-{}^{12}C^{1}H_2$ group is 14.0078, while the precise mass of ^{14}N is 14.0031. These differences from integral values are generally ignored in presenting mass spectra graphically, but can be used to determine the elemental composition of each ion if an instrument of sufficiently high resolution is available. This may be of great value when it is necessary to infer the probable structure of an unknown compound from its mass spectrum.

A number of excellent texts on mass spectrometry can be recommended to the reader unfamiliar with the field (17–21). The remainder of this review will be concerned with technological developments of particular interest in the context of pharmacology, and with specific applications of GC/MS to problems of pharmacological and toxicological interest.

Technological Developments

Molecule separators.—The molecule separators most commonly used in pharmacological studies are the Becker/Ryhage two-stage jet separator (22, 23) which is an integral part of the LKB 9000 GC/MS system, and the glass frit separator originally described by Watson & Biemann (24) or a modification of it (25). It is well known that irreversible losses of trace components may occur in GC systems that allow the sample to contact a hot metal surface; for this reason separators based on effusion through porous stainless steel or silver (13) do not appear well adapted to pharmacological and biomedical applications in which the sample may be both extremely small and labile. Membrane separators of various kinds have been described (13, 26) and a recent version (27) appears particularly well adapted to pharmacological studies because of its simplicity of design and all-glass construction. Membrane separators are based on the high permeability of organic compounds through a silicone elastomer membrane relative to the inorganic carrier gas (He or N_2), which exists at atmospheric pressure. No supplementary vacuum pump is needed in the single stage versions (26, 27).

A recent series of innovations in molecular separators (28–31) appears particularly promising because they approach the theoretical ideal of complete removal of carrier gas and complete transmission of sample. The principle involved is the selective diffusion of hydrogen, which is used as a carrier gas, through a palladium/silver alloy tube, to be removed either electrolytically or by combustion. The only significant drawbacks of this approach appear to be a catalytic reduction of some types of compound in the separator, and temporary breakthrough of hydrogen when the separator is exposed to sulfur-containing compounds.

Ion sources.—Until recently, practically all pharmacological studies with GC/MS used electron impact (EI) ionization, in which the incoming molecular

stream encounters a beam of electrons from a hot filament with controlled energy (usually 70 eV). Both positive and negative ions are formed, of which only the former are generally analyzed. Many other means of ionization have been introduced, and have recently been reviewed (32–34). One of the disadvantages of EI ionization is that the molecular ion may have a very low abundance and sometimes is not detectable. Since the mass of the molecular ion indicates the molecular weight of the compound, its absence makes identification more difficult. Although the relative abundance of the molecular ion may be increased by operating the source at a lower electron energy, the sensitivity is also greatly reduced. Alternative approaches are to use field ionization (35) or chemical ionization (CI), of which the latter appears considerably more promising (36–40). In chemical ionization MS, the sample is mixed with a relatively large quantity of a reactant gas in a specially designed ion source, which is operated at a very high pressure (up to 5 mm). In a GC/MS system, the reactant gas (commonly methane, isobutane, or a noble gas) can serve as a GC carrier gas and a molecular separator is not used. The electron beam produces ionization of the reactant gas, which is present in very much higher partial pressure than the sample, and this in turn causes ionization of the sample, generally by adding a proton or abstracting hydride ion to give $(M + 1)^+$ or $(M - 1)^+$ (quasimolecular ions). The resulting spectra differ in having relatively more abundant ions in the higher mass range, including the quasimolecular ion, much greater simplicity (fewer fragments), and independence from some secondary factors (electron energy, accelerating voltage, source pressure). A GC/MS/CI system with isobutane as carrier and reactant gas has been used to identify barbiturates, which frequently lack a detectable molecular ion on EI ionization (39), and to differentiate structural isomers (41). The greater simplicity of CI spectra makes it desirable to provide for easy selection of CI or EI on the same instrument; a modification of the EAI Quad 300 has been described to accomplish this (42).

Mass analyzer.—Design requirements for a mass spectrometer to be used as part of a GC/MS system have been considered in detail elsewhere (12). Most modern mass spectrometers can be equipped with a gas chromatographic inlet, and used as an integrated system, but pharmacologists will generally be concerned only with complete commercial systems. These are of two main types:

1. Magnetic sector instruments in which the ions are separated according to their m/e by passing through a magnetic field. For historical reasons most of the pharmacological literature on GC/MS has involved the use of instruments of this type.

2. Quadrupole mass spectrometers in which ions formed in the source pass into the long axis of four parallel rods, to which a programmed combination of RF and DC voltages is applied. Ions of only one mass/charge ratio maintain a stable trajectory and reach the detector at the other end; others have unstable trajectories and do not reach the detector.

Each type of instrument has its advantages. The magnetic instruments have a higher mass range and higher resolution; a device has been described to allow

high resolution data to be obtained from a medium resolution instrument (43), providing the information necessary to calculate elemental composition. In contrast, the mass range of most quadrupoles is limited to 500, and it is only possible to measure nominal masses of ions. Most compounds of pharmacological interest have molecular weights below 500, and this mass range is therefore sufficient provided that derivatization does not result in too large an increase. For example, multiple substitution with trimethylsilyl (mass 73) or trifluoroacetyl (mass 97) groups may easily exceed this limit. Quadrupole mass spectrometers are generally much less expensive, more compact and less demanding of service than magnetic instruments. The m/e on which the instrument is focussed is linearly related to a control voltage, and the m/e axis of a recorded scan is linear, unlike a magnetic instrument in which the mass spacing becomes smaller as the mass scale is ascended. This is not important when a dedicated computer system is used, but is convenient for visual interpretation of an oscillographic record. A quadrupole instrument is easier to program discontinuously for specific ion detection (see below) and is inherently more stable and versatile than a magnetic instrument when used in that mode (44–46). Finally, a quadrupole is relatively tolerant of higher operating pressures, which makes it more readily adaptable for CI mass spectrometry.

Detection and output devices.—The relevant mass range must be scanned sufficiently fast so that the composition and concentration of the sample does not change significantly during the scan (unless subsequent corrections are made in data reduction). The ever-present need to achieve the maximum possible sensitivity dictates the use of a low-noise detector. An electron multiplier followed by a high-speed electrometer (up to 10^4 Hz) is necessary to meet these requirements. Ion-counting electronics have obvious advantages in this application because of the very low noise level and elimination of a subsequent analog/digital (A/D) conversion step for data reduction. Limitations of dynamic range may explain why this system is little used at present.

The output devices normally available are:

1. A high-speed oscillographic recorder with several channels that record the electrometer output at different sensitivities. This is used to record complete scans of the mass spectrum in periods of 0.5–5 seconds.

2. A multichannel potentiometric recorder, used to monitor the ion current either from specific masses or integrated over-all masses (total ion current monitor). This is required to monitor the GC effluent to determine scan times, and to estimate isotope ratios (see below "Specific Ion Detection").

3. An oscilloscope is generally used to monitor the mass spectrum on a time of flight or quadrupole instrument, which is capable of scanning the complete mass range 10–10^3 times per second.

4. Magnetic tape is often used to record GC/MS output either digitally or in analog form for subsequent analysis by computer.

5. On-line data reduction by a small dedicated computer, with the results presented on a scope display, teletype or plotter.

Data processing.—Because of the enormous rates of data generation by a GC/MS system (up to 10^4 bytes/second), it is not possible to use more than a small fraction of the available information unless automatic data processing equipment is available. The equipment required depends on the uses to which an individual GC/MS system will be put and the modes of operation in which it will be most frequently employed. Applications of computers to mass spectrometry and GC/MS have recently been reviewed (47, 48). They may be categorized as follows:

1. One-line processing of data to provide normalized mass spectra from single or repetitive scans, with or without editing, display, background subtraction, and optional tape storage capability (49–54).
2. Analysis of data stored on magnetic tape, drum, or disc to recover specific spectra or "mass chromatograms" (graph of a specific ion abundance against time) (55–57).
3. Comparison of a mass spectrum with a reference file of spectra to identify compound(s) with similar or identical spectra (49, 58–64).
4. Interpretation of mass spectra to provide ab initio information about the compound that generated it (64–66).
5. Deconvolution of overlapping gas chromatographic peaks (67–70).
6. Time averaging of repetitive scans for signal/noise enhancement (71).

APPLICATIONS

Sample preparation and chromatography.—Sources of samples to be analyzed by GC/MS in pharmacological research are generally tissues or biological fluids. Preliminary purification steps are necessary to remove proteins, sugars, lipids, and other endogenous compounds that would otherwise be deposited in the injection port, where decomposition would result in contamination of the entire system. The usual approach is to extract the fluid or homogenate with a polar organic solvent such as ethyl acetate (72) or chloroform (61, 73), and examine the GC peaks in this fraction. Niyogi & Rieders (74) have described some of the interfering endogenous compounds that are extracted by chloroform, and their gas chromatographic properties. Other workers have employed the less polar hydrocarbon solvents to isolate drugs and their metabolites from in vitro incubation mixtures (75). In general it is preferable to use the less polar solvents to minimize the extraction of endogenous compounds.

If the properties of the substance to be extracted are known, purification and concentration can be achieved by a series of extractions. For example, ketamine can be extracted from plasma to heptane, but can be concentrated about 100-fold by a back extraction into a small volume of HCl followed by reextraction into a smaller volume of dichloroethane (76). Other acidic and basic compounds can be concentrated and purified in this manner using their solubility in ionizing aqueous media and back extraction into another organic solvent.

For gas chromatography, polar functional groups such as OH, NH, and COOH are frequently derivatized by acylation or alkylation to minimize adsorption on the gas chromatographic column. Adsorption causes losses that may result in reduced sensitivity, poor resolution, and even complete disappearance of

very small samples. In some instances, derivatives such as the heptafluorobutyryl function are chosen in order to enhance electron capture and thereby increase sensitivity when using electron capture detection. Gas chromatographic requirements for MS detection are similar to those for most other types of detection, so that derivatization is frequently necessary. However, as stated in the section on mass analyzers, the molecular weight of the derivative should not be unnecessarily increased if the mass range of the available mass spectrometer is limited. Derivatives such as the heptafluorobutyryl amide or ester may not be appropriate, and generally have no obvious advantage over shorter chain derivatives. The trimethylsilyl (TMS, $(CH_3)_3Si$-) group is also used to mask $-OH$ and $-NH$ groups, but because of its high formula weight (F.W. = 73) it would increase the molecular weight of a polyhydroxy compound beyond practical limits of spectrometers with limited mass range. The function has been used to convert N-hydroxyphentermine (2-phenyl-1,1-dimethylethyl hydroxylamine) to a volatile derivative for a GC/MS study to determine its structure (77) and for the gas chromatography of amino acids (78). Trifluoroacetic anhydride has frequently been used in GC/MS studies (79). This compound is highly reactive and readily acylates primary and secondary amine groups. In a GC/MS assay for amphetamine (80, 81) the N-trifluoroacetyl (TFA) derivative was used because of its GC column characteristics and because a base peak in the 100–200 mass range was desirable. The n-butylboronate is a derivative that should be of considerable interest to pharmacologists (82). This derivative is formed by treatment of 1,2 aminoalcohols and 1,2 diols including catecholamines with n-butylboronic acid in pyridine. The resulting mixture can be directly injected into the GC/MS. The derivatives are readily chromatographed on a 1% OV 17 column at 190° and give in some instances large quantities of the molecular ion (83). Other derivatives that have been used in GC/MS work include the isothiocyanate derivatives of primary amines (84), methyl esters of carboxylic acids (85), and O-methoxime derivatives of prostaglandins (86) and steroids (87).

The gas chromatographic conditions employed in GC/MS studies have not been unique or unusual. One major problem with a coupled GC/MS system is "bleeding" of liquid phase into the mass spectrometer, resulting in a "background" mass spectrum of the liquid phase components. For this reason, the more stable liquid phases are preferred and the column should be operated at relatively low temperatures (<200°). Criteria for the choice of gas chromatographic columns for gas chromatography in general have been discussed (2).

Identification of compounds.—To establish the identity of a compound rigorously by GC/MS, it is necessary to show that its mass spectrum is the same as that of an authentic reference compound recorded in the same system under identical conditions. Presumptive evidence may be obtained by inspection or by comparison with a reference file of mass spectra accumulated from the literature, but a number of factors may influence the mass spectrum and vitiate the comparison. These include the type of instrument, type of ion source, electron energy, and temperature of the ion source. Many spectra in reference files were obtained with

a direct inlet system and the compounds were of questionable purity; on the other hand spectra obtained with a GC/MS system may be contaminated with column bleed. For these reasons it is necessary to compare spectra of unknown and reference compounds obtained in the same system under the same conditions.

A spectrum is usually obtained by scanning at the maximum of a gas chromatographic peak sufficiently fast so that the concentration does not change significantly during the scan. The background spectrum must also be recorded and subtracted and the results are then normalized so that the base peak has an abundance of 100%.

Applications of GC/MS to the structural identification of drugs and related compounds have been in three general areas: forensic analysis, clinical analysis, and drug metabolism. Nakamura et al (88) have analyzed "street heroin" by comparing the mass spectra obtained from a GC/MS analysis of a methanol solution with authentic heroin. The presence of procaine in the sample was also demonstrated. In studies with 13 poisoning cases, Blomquist & co-workers (73) identified depressants, stimulants and solvents in blood and tissues of autopsy specimens from chloroform extracts of the biological material. The spectra of other compounds of forensic interest such as LSD (89), other hallucinogens (90, 91) and parathion (92) have been reported.

In an application of GC/MS to a clinical problem, the urine of a patient suspected of ingesting an overdose of chlordiazepoxide was examined and shown by examination to contain propoxyphene (93). A urine extract was injected into the GC/MS and the mass spectra of the major gas chromatographic peaks determined. Chlordiazepoxide was readily eliminated as a possibility because it contains chlorine that is readily recognizable by the natural abundance (3:1) of its ^{35}Cl and ^{37}Cl isotopes. The presence of benzyl alcohol and diphenylbutene in the chromatogram indicated another ring system. Mass spectra of individual gas chromatographic peaks were analyzed by a computer system that searches a library of mass spectra. When several of the GC peaks were analyzed by this technique the tentative identification of propoxyphene was made which was subsequently identified as one of the GC peaks. The other GC peaks analyzed were metabolites of propoxyphene. It should be pointed out that the structural elucidation was carried out by the NIH sponsored Special Research Resource Facility for Mass Spectrometry at MIT and could not be done without extensive collaboration between clinical and basic science groups. Similarly a group at the NIH has established a GC/MS computer system to analyze material present in biological fluids such as gastric lavage or blood collected from overdose patients (61). In their routine an extract of the biological fluid is evaporated and the residue injected into the GC column where the mass spectra of the different GC peaks are collected and analyzed using a computer. The computer scans the five largest peaks in the mass spectrum and compares the relative intensities with spectra on file. The gas chromatographic retention time is also useful in confirming identity. Under appropriate conditions these determinations can be achieved in a minimum of time so that the information obtained can be used as the basis for treatment.

Identification of metabolites is a simpler problem than the identification of a drug in an overdose case since the structure of the parent drug is known and likely structures of metabolites can be predicted. The procedure commonly employed for identification of metabolites formed in vivo is to examine an organic extract of plasma or urine. The extract can be concentrated or purified further and in some instances it is treated with a derivatizing reagent to enhance the chromatographic separation of the mixture. The gas chromatogram is then monitored by total ion current and the mass spectrum of each GC peak is scrutinized for fragments characteristic of the parent drug or a postulated metabolite. The identity of possible metabolites is tentatively established by inspection and/or reference to a file of spectra, and must then be confirmed by comparison with authentic compounds. GC/MS has also been used to examine metabolism in vitro. The value of GC/MS in metabolite elucidation can be best shown by comparing two studies conducted by the authors. The first was the identification of oxotremorine as the active metabolite of tremorine, which was based on studies with a crystalline salt of a metabolite isolated from several pooled incubations of volumes of five liters with liver slices (94). More recently, N-hydroxyphentermine, a metabolite of phentermine, has been identified with GC/MS procedures using the extract of a 6 ml incubation mixture (77). In other applications Daly (95) studied the metabolism of some acetanilides and anisoles with rat liver microsomes and used GC/MS to examine the phenolic metabolites formed. The metabolites were isolated by thin layer chromatography and the TLC scrapings were extracted with ethylacetate for injection into the GC/MS. In this way, several hydroxylated metabolites of halogenated anisoles and acetanilides were identified. GC/MS has also been used in the identification of 2,4,6-trimethylacetophenone oxime as the metabolite of 2,4,6 trimethylacetophenone imine. The metabolite was identified in ethyl acetate extracts of incubates from both rat and rabbit liver microsomes (85). The metabolites of prolintane [DL-1-(α-propylphenethyl)-pyrrolidine] were examined in vitro and the major metabolite was identified as the corresponding pyrrolidone (96). In this case however, instead of using a GC/MS system, the metabolite was isolated on a thin-layer chromatographic plate and the spot was eluted off and extracted into heptane. The metabolite was isolated from the heptane solution and examined directly with a mass spectrometer.

Metabolite identification studies performed by these techniques are included in Table 1. Tables 2 and 3 list reports in the literature in which GC/MS was used to identify other compounds of pharmacological and toxicological interest. Earlier literature is covered in the review by Guarino & Fales (10).

Specific ion detection.—If a mass spectrometer is focussed on a fixed mass/charge ratio instead of scanning, it behaves as a highly specific gas chromatographic detector, responding only to compounds that, on fragmentation, yield ions of the specific mass upon which it is focussed. Both the chemical noise (i.e. interfering compounds giving a detector response) and random statistical noise are reduced relative to the signal, and the effective sensitivity of the instrument

TABLE 1. Drugs and Their Metabolites Identified by GC/MS

Substances	References
Aminopyrine and metabolites	97
Antipyrine and metabolites	98
Barbiturates and metabolites	39, 99, 100
Cambendazole metabolites	101
Chlorpromazine metabolites	102
N-Cyclopropyl-2-chlorophenoxyethylamine and metabolites	103
1-{*o*-[[3-dimethylamino)propyl]thio]phenyl}-3-methyl urea (metabolites)	104
Diphenylhydantoin and metabolites	105, 106
Hashish constituents	107
Lidocaine metabolites	108
Mescaline	109
Nortriptyline and metabolites	110, 111
Oxprenolol and metabolites	112
Phentermine metabolite	77
Probenecid	113
Prolintane metabolite	96
Propoxyphene	93
Propranolol and metabolites	114
Reserpine	115
Sympathomimetic amines and metabolites	79, 84, 116, 117
Tetrahydrocannabinol and metabolites	118, 119
Tremorine and metabolites	120, 121
Triallyl phosphate	122
2,4,6-Trimethylacetophenone oxime	85
Drugs of abuse	61, 88, 123
Psychoactive tryptamines	124

is correspondingly enhanced. In practice it is generally possible to detect a few picograms ($\sim$10 pg) under favorable circumstances. This technique has been called "mass fragmentography" (102) and has been pioneered by Holmstedt, Hammar and their colleagues (43, 102, 121, 126, 162–164). The advantages of scanning and specific ion detection are to a large extent retained when a programming device is available that allows the mass spectrometer focus to be discontinuously switched between a few masses known to be particularly informative in a given context. This extension of "mass fragmentography", also known as "multiple specific ion detection" (43, 45, 46, 162), retains comparable sensitivity but allows more definitive identification of the compound responsible for a gas chromatographic peak by measurement of the relative abundance of a few important ions and comparison with a standard. It was first employed by Sweeley & co-workers (165) to resolve overlapping gas chromatographic peaks,

TABLE 2. Endogenous Substances Identified by GC/MS

Substances	References
Acetylcholine	125–130
Amino acids	78, 131, 132
Cardiolipin	133
Constituents of breath	134
Dopamine	135
Estrogens	136
Homovanillic acid	137
5-Hydroxytryptamine	138
Indoleacetic acid	139
Lipids	140, 141
Phosphatidyl cholines	142
Prostaglandins	143
Pteridines	144
Pyroglutamic acid in urine	145
Steroids	56, 87, 146–148
Tyramine	149
Urinary acids	146, 150, 151
Urinary components in metabolic disease	152

TABLE 3. Food Additives and Environmental Poisons

Substances	References
Alfalfa aroma	153
Chlorinated biphenyls	154
Chromium and beryllium	155
Garlic principle	156
Nitrosamines	157, 158
Pesticides	159
Phosphates and phosphonates	160
Pungent principles of *Capsicum annuum*	161

using an accelerating voltage alternator (AVA) on the LKB9000. Subsequent work has extended the versatility of the AVA (43, 162) to 4 masses over a 20% range; similar devices have been described that allow quadrupole instruments to be used for up to 8 masses over the entire mass range, with separate demodulated outputs (44–46). Applications of multiple specific ion detection have been reviewed (102, 162, 166). It has been successfully applied to the analysis of steroids (146, 166), acetylcholine (126), serotonin (138), melatonin and 5-methoxytryptamine (167), p-tyramine (149, 168), indole-3-acetic acid (139), prostaglandins (169), chlorpromazine and metabolites (102, 170), nortriptyline and

metabolites (163, 171), amphetamine (81), phenmetrazine and related amines (116), tetrahydrocannabinol and metabolites (105, 172), N-hydroxy-phentermine (77) and nitrosamines in food (173), in addition to the compounds discussed in the next section in which quantitation was achieved by using internal standards labelled with stable isotopes.

A mass fragmentogram (102) and a mass chromatogram (55) present essentially the same types of information: both represent the abundance of an ion of a specific mass as a function of time in the gas chromatographic effluent. The difference lies in the procedure used to obtain them. A mass chromatogram is calculated either on-line or from data stored magnetically by repetitive scanning of the spectrum during a gas chromatographic analysis. No previous knowledge of the masses of interest is required. A mass fragmentogram is obtained by on-line analog processing of data, and requires that the mass spectrometer is focussed beforehand on the masses at which the ion abundances are to be measured. The advantage of this approach (apart from the relative cost of the equipment required) lies in the much higher signal/noise ratio that can be obtained, and a correspondingly lower limit of detection. This derives basically from the fact that during a 1 sec scan over a mass range of 500 amu, the ion abundance at any one mass is measured for less than 2 msec, whereas in fixed focus mode the entire period (1 sec) is used to monitor the ion of interest. Statistical noise decreases in relation to the signal by a factor proportional to the square root of the averaging time; hence the substantial enhancement of signal/noise ratio in fixed focus mode.

Quantitative measurements.—For accurate quantitative measurement of a compound by gas chromatography it is necessary to use an internal standard which is added to the sample to be analyzed, and which has a retention time close to the compound to be measured. Quantitation is based on the ratio of the peak heights, with reference to a standard curve relating peak height to mole ratio. In this way it is possible to avoid or substantially reduce errors arising from losses during work-up and chromatography (2, 3). The ideal internal standard should be as similar as possible to the compound to be analyzed, so that losses of the two are equal; yet it must be capable of separate measurement. In conventional gas chromatography this is generally achieved by using as an internal standard a homolog or closely related compound with a slightly different retention time. The same technique has been successfully used for mass spectrometric detection of dopamine and norepinephrine [as pentafluoropropionate derivatives (173a)], indoleacetic acid (as the heptafluorobutyryl, methyl ester derivative) (139), and nortriptyline (as the trifluoroacetamide) (163, 171). A GC/MS system in specific ion detection mode offers another more ideal possibility: the use of an isotopically labelled version of the same compound as an internal standard. The fragmentation pattern and retention time will be the same (unless there are isotope effects) but some fragments will contain one or more atoms of an isotope such as deuterium rather than the more abundant natural isotope. These fragments will be shifted on the mass scale by one or more atomic mass

units (Figure 1) and one or more can be simultaneously measured by other channels of the specific ion detector. The analysis depends on the measurement of an isotope ratio of a single gas chromatographic component with the mass spectrometer, and is essentially an isotope dilution technique. If the internal standard is added in large excess (10–100×), it will also serve as a carrier and minimize adsorption losses, which are extremely troublesome in gas chromatography in the nanogram range.

This technique was first employed by Gaffney et al (174) who studied isotopic variants of nortriptyline labelled in the side-chain as $=CHCH_2C^2H_2NHCH_3$ and $=CHCH_2C^2H_2{}^{15}NHCH_3$, giving molecular ions with masses increased by 2 and 3 atomic mass units respectively. The compounds were analyzed as TFA derivatives. The behavior of these variants as internal standards was superior to that of a related compound that had previously been used, especially as the detection limit was approached (<100 pg), when differential loss of the non-isotopic internal standard was seen. An analogous method for quantitative estimation of prostaglandin E_1 by GC/MS was described by Samuelsson et al (143); however, in this procedure the deuterated internal standard was added after conversion to the methyl ester and O-methoxime derivative (86). The $^2H_3$ label was in the methoxime group of the internal standard, which was present in an excess of 100-1000:1, and therefore served also as a carrier. Monitoring at m/e 470 and 473, the lower limit of detection was 3 ng. Carriers that are already labelled in the parent compound have the advantage that carrier can be added before work-up derivatization, and also offer a choice of derivatives for chromatography. Quantitative GC/MS methods for analysis of prostaglandins $F_{2\alpha}$ and E_2 have since been described (175), based on the 3,3,4,4-$^2H_4$ variants as carriers and internal standards. Prostaglandin E_2 was converted to the diacetate, O-methoxime methyl ester, and prostaglandin $F_{2\alpha}$ was analyzed as the triacetate methyl ester. The limit of detection was 250 pg. Alternative methods of derivatization have been recommended (169, 176). Similar methods using deuterium-labelled internal standards have been described for choline and acetylcholine (after N-demethylation) (128), amphetamine (as the TFA derivative (80, 81), 5-hydroxyindoleacetic acid (as the bis[heptafluorobutyryl] methyl ester derivative) (177), and β estradiol and estrone (as the TMS ethers) (178). Homovanillic acid has been estimated (after conversion to the methyl ester) using a labelled derivative (O-$^2H_3$-methyl) as internal standard, with subsequent conversion to the heptafluorobutyrate (137).

The position at which the isotopic label is introduced is of considerable importance, and must be decided upon the basis of the fragmentation mechanism. The label must be retained in an ion of relatively high abundance in order to maximize sensitivity, and the ion chosen for the analysis should be isolated on the mass scale from other ionic fragments so that each of the two masses selected (derived from endogenous compound and labelled standard respectively) is as far as possible representative of one isotopic variant, and is not produced by the other. Since any unlabelled variant present in the internal standard will appear as a blank, it is likely to limit the sensitivity of the procedure by providing a

statistically variable background, and the internal standard should therefore have the maximum possible isotopic purity. This is particularly important in low-level work, where the internal standard also serves as a carrier and may be present in 100–1000-fold excess.

For labelling of internal standards deuterium has many advantages over other stable isotopes. Deuterium-labelled chemical intermediates are available in greater variety, lower cost, and higher isotopic purity than those of any other suitable nuclide, and it is generally possible to introduce several ^2H atoms into each molecule. This allows lower contamination of the internal standards with the unlabelled variant and hence reduces the background. In most of the studies referred to above, the background contributed by the internal standard at the mass chosen for measurement of the unlabelled variant was 0.1–0.5 %, and may be partly accounted for by ions of the same mass but different structure.

These procedures emphasize the need for accurate means of isotope ratio measurement, and several techniques have been described using magnetic instruments (43, 165, 179–181) and quadrupoles (44–46). The systems described by Klein et al (179, 180) and by Frew & Isenhour (181) are designed for the precise measurement of an isotope ratio in which the abundance of one of the ions may be very small. They are therefore well adapted to the stable isotope dilution methods described above, or for estimating the dilution (i.e. specific activity) of a stable isotope labelled tracer (see below). The older accelerating voltage alternator (165) and its recent modification (43) provide for measurements of three and four different ions simultaneously within a specified mass range (10% and 20% respectively), and have been widely used for multiple ion detection. Quadrupole devices (44–46) have been described more recently and have so far been little used, although up to eight channels are available over the whole mass range of the instrument. Discrete smoothed outputs are provided for each mass, with separate attenuation, bucking, and integration capability (45, 46).

Other applications of stable isotopes in GC/MS.—Stable isotopes have for many years been used as tracers in biochemical studies (182). With their readier availability, greater purity, lower cost, and better methods for routine measurement, they are now being increasingly used to solve pharmacological problems (179, 183, 184), not only as internal standards for quantitative GC/MS analysis but as tracers, and to provide recognizable mass spectral signals for the identification of metabolites. GC/MS appears to be emerging as the only method of accurate stable isotope measurement presently available with the sensitivity frequently required for studies of drug metabolism.

There are scattered reports of significant isotope effects in pharmacology (185–188), mainly with deuterium. Stable isotopes of carbon, nitrogen, and perhaps oxygen may be preferable to deuterium for labelling of compounds to be used as tracers (as opposed to internal standards), since isotope effects are less likely to be important, and the label is much less likely than deuterium to undergo random exchange.

Knapp, Gaffney & colleagues (110, 111, 183) have used mixtures of equal

amounts of labelled and unlabelled variants of nortriptyline in order to create recognizable clusters (in this case doublets) of mass spectral peaks in its metabolites. The label was an N-C^2H_3 group which, as the authors state, was unfortunate because this group is rapidly removed by metabolism. A preferable site for labelling would have been the ring system. A similar application has been reported using a ^{15}N label to study metabolism of a barbiturate (189, 190). The artificial clusters are analogous to those created by the natural isotopic abundances of chlorine and bromine, and provide a clue for the recognition of chromatographic peaks which represent metabolic transformation products of the labelled parent compound.

Stable isotopes have been more frequently used as quantitative tracers (see several examples in 184). They have obvious advantages over radioisotopes, particularly in clinical use, where radiation hazards are not acceptable (especially in children and pregnant women). The chief limitation in the use of stable isotope tracers has been the availability, cost, and sensitivity of the necessary instrumentation for measurement. Present GC/MS systems are capable of providing measurements of isotope ratios comparable to the limits of useful detection of ^{14}C. Danzinger et al (191) have recently compared ^2H and ^3H labelled bile acids for the study of bile acid kinetics in man. By simultaneously using different isotopic variants as an internal standard and a tracer, it is possible in one analysis to estimate not only the quantity of tracer but also the quantity of unlabelled compound in the sample. This technique has been employed in studying the kinetics of acetylcholine and choline metabolism (Jenden, D. J., unpublished data).

In contrast to radioisotopic methods, GC/MS allows the separate measurement of many isotopic forms of the same compound, by distinguishing the position of the label within the molecule. Since only two radioactive labels (^3H and ^{14}C) can generally be used in biochemical pharmacology because of the nature of the compounds under study, stable isotope labelling opens new opportunities in multiple labelling experiments to study kinetic systems.

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