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REVIEW

THE PLACE OF GAS CHROMATOGRAPHY — MASS SPECTROMETRY IN CLINICAL CHEMISTRY

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CONTENTS

1. INTRODUCTION

Mass spectrometry (MS) in its modern and advanced form has been used for approximately ten years in clinical and clinically related laboratories. However, it should not remain unmentioned that MS techniques have been applied in clinical research for more than twenty years in the analysis of respiratory and blood gases. It was employed in lung function tests to determine nitrogen, oxygen and carbon dioxide in expired air, sampled over a mouth piece, and to perform regional analyses within the bronchial tree in diagnostic bronchoscopy [1]. The technique is suitable for instantaneous and continuous gas measurements. For continuous in vivo determinations of O_2- and CO_2-pressures in animals the gases were sampled from circulating blood through a permeable membrane at the tip of an intravascular cannula which was connected to the mass spectrometer [2]. In general, the instruments used for respiration gases are built for small mass ranges, e.g. *m/e* 18—80 [2] and have low resolution. In spite of the potentials of the technique for gas analyses MS has not been widely

accepted in this area. The reasons for this were summarized by the following comment in The Lancet in 1960: "Unfortunately a mass spectrometer is a formidable instrument, only likely to be found in specialized laboratories" [3]. Even today many clinical chemists are reluctant to use MS.

The object of this paper is to attempt a brief survey of the potentials and the current situation of MS in clinical chemistry and clinical biochemistry. To present a complete review of the subject would exceed the scope of the paper. Only the main aspects shall be outlined, and technical questions cannot be discussed. A recent and detailed review has been written by Lawson [4]. Since the majority of the problems in clinical chemistry using MS apply to its coupling with gas chromatography (GC), GC—MS combination will be emphasized.

GC is a method used for separating volatile compounds and all substances which can be made volatile by derivatization. Because of its flexibility and broad applicability it has been, until now the most frequently used analytical separation method. No other method equals GC in separating power and efficiency. Using calibration curves and internal standards, quantitative analyses with high precision are possible.

MS is an analytical technique for: (i) identification of unknown substances; (ii) sensitive and highly specific detection of known substances.

For the clinical chemist the combination of GC and MS can be an almost ideal analytical system for the following problems:

(i) Identification of unknown substances of low concentration in complex mixtures of biological origin. Depending on the chemical properties of the substances 10—100 ng of injected material are sufficient for identification.

(ii) Specific qualitative detection of known substances. The detection limit is 1—100 pg.

(iii) Specific quantitative determination of known substances. Depending on the chemical nature of the substance, the detection limit is approximately 5—100 pg.

(ii) and (iii) are achieved by mass fragmentography (MF), also called selective ion monitoring (SIM), either by single ion detection (SID) or multiple ion detection (MID).

Substances which cannot be made volatile are not amenable to analysis by GC—MS. For such substances the combination of liquid chromatography with MS would be a most valuable addition to GC—MS.

Considering the efficiency of the GC—MS system it appears understandable that until now the following attributes are still not completely eliminated from GC—MS: high costs, complexity, need for skilled personnel, frequent maintenance requirements.

By attaching a computer to the GC—MS system (GC—MS—COM), the potentials are enhanced considerably, and the following operations become feasible:

(i) Automatic repetitive scanning, enabling a more complete and less tedious analysis of complex profiles.

(ii) Storage and documentation of the MS data, with the possibility of presentation on display or plotter.

(iii) Spectrum transformations, background subtraction and further manipulations to facilitate spectrum identification.

(iv) Elemental composition analysis using high MS resolving power, to give additional information on unknown substances.

(v) Computer matching of recorded spectra against files of reference spectra.

(vi) Computer MF, enabling selective detection of various, not pre-selected compounds and classes of compounds after a single GC—MS run. Spotting of known, and searching for predicted, substances.

In Fig. 1 the GC—MS—COM system of our laboratory is schematically represented. Similar configurations are used elsewhere. An example of a GC—MS—COM analysis of volatile metabolites in urine using automatic repetitive scanning is given in Fig. 2.

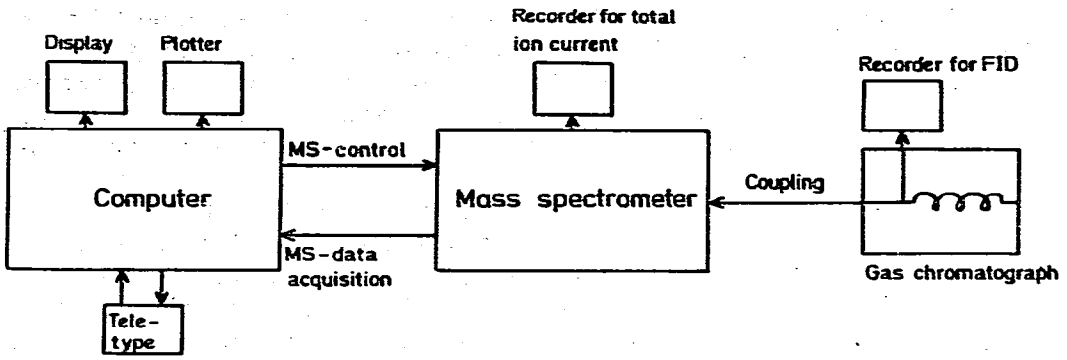

Fig. 1. Configuration of a GC—MS—COM system.

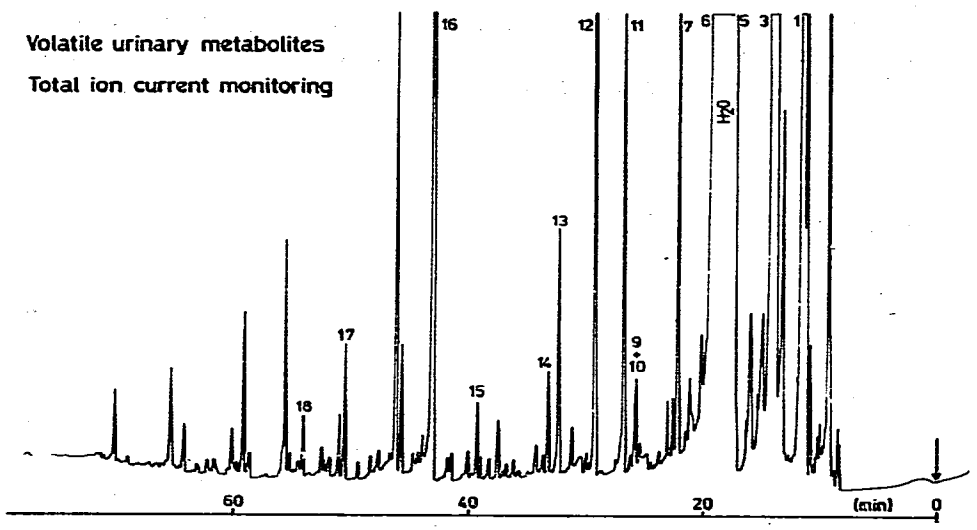

Fig. 2. GC—MS analysis of volatile metabolites in urine. 1 = Acetone, 3 = ethanol, 5 = 2-pentanone, 6 = *n*-propanol, 7 = dimethyl disulfide, 9 = 3-penten-2-one, 10 = N-methylpyrrole, 11 = *n*-butanol, 12 = 4-heptanone, 13 = isopentanol, 14 = 2-heptanone, 15 = cyclohexanone, 16 = allyl isothiocyanate, 17 = pyrrole, 18 = benzaldehyde. From J. Chromatogr., 112 (1975) 539.

The enormous number of applications of GC—MS and GC—MS—COM shall be classified in four areas:

I. Structural identification of substances.

II. Profile analyses and pattern recognition.

III. MF determinations and reference methods.

IV. In vivo experiments with substances labeled with stable isotopes.

2. STRUCTURAL IDENTIFICATION OF SUBSTANCES

More than any other analytical technique MS is suited to the identification of substances. In simple compounds the molecular ion and fragmentation pattern enable the analyst to identify the substance. As an example (Fig. 3), the molecular ion *m/e* 114, the fragment ions *m/e* 43 and *m/e* 71, and the McLafferty rearrangement ion *m/e* 86, together with the GC retention time suggest peak number 12 to be 4-heptanone. Computer matching against files of reference spectra and elemental composition analysis are aids in identifying more complicated molecules.

To a large extent structural identifications by GC—MS or GC—MS—COM have been performed on urine, plasma and tissue steroids in normal newborns, infants and adults [5—13] and in patients e.g. with adrenal malfunctions and enzyme defects [14—16]. The steroids are analyzed as derivatives such as trimethylsilyl ethers or methoximes—trimethylsilyl ethers. Packed glass columns originally used for steroid analyses, have been almost completely replaced by glass capillary columns, introduced for instance by Grob [17], as having much higher separation efficiency [11, 18, 19]. Not only for steroids but for practically all other complex biological mixtures, capillary columns should be used instead of packed columns.

A great multitude of organic acids and amino acids have been identified in body fluids and tissues [20—24]. Many of these studies are connected with investigations of metabolic abnormalities and inborn errors of metabolism,

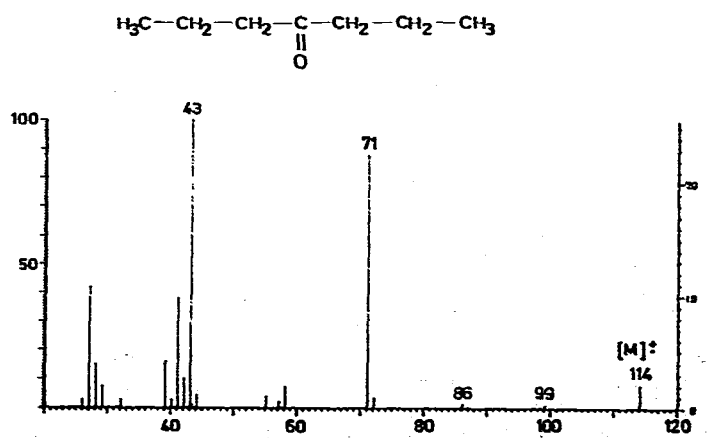

Fig. 3. Mass spectrum of peak number 12 in Fig. 2, identified as 4-heptanone.

such as congenital lactic acidosis [25, 26], ketoacidosis [27], propionic and methylmalonic acidemia [28], β-methylcrotonylglycinuria [29], dicarboxylic aciduria [30] and the corresponding enzyme deficiencies. Due to the great amount of GC and MS data on acids and other metabolites, it has been suggested by Jellum [107] and by Macek [31], that the data may be coordinated on an international basis.

Metabolites which are volatile without derivatization, such as ketones, alcohols, sulphur compounds and furans are especially amenable to GC—MS analysis, and were identified in urine and blood [33—36].

Considerable achievements were made in the structural identification of the different types of prostaglandins and their metabolites. Methyl ester—trimethylsilyl ether—methoxine derivatives enabled the determination of the hydroxyl- and keto- groups and of the double bond positions [37].

In the analysis of very complex molecules, such as free ceramides in human platelets [38], ceramides from plasma sphingomyelin [39], oligosaccharides in urine [40, 41] and glycosphingolipids with blood group specificity [42—44] MS is indispensable. Sequence and bond positions in oligosaccharides and glycosphingolipids could be established. Mass spectrometric sequencing was also used in peptides [45] by analyzing di-, tri- and tetrapeptides obtained by partial hydrolysis. The primary structure of ovine hypothalamic luteinizing hormone-releasing factor (LRF), a decapeptide, was established using GC—MS [46].

3. PROFILE ANALYSES AND PATTERN RECOGNITION

GC—MS is most powerful for profile analyses and recognition of normal and abnormal patterns of biological substances in body fluids and tissues. Whereas conventional clinical chemical tests determine individual substrates, hormones or groups of hormones which are known to be present, profile analyses offer the opportunity to:

(a) detect and characterize a large number of substances simultaneously;

(b) find new and unexpected substances;

(c) determine changes in the ratios of different constituents;

(d) give much more precise information, e.g. on steroid hormones, than a group test.

Abnormalities in urinary or blood steroids may be overlooked by a group test or by radioimmunological determination of one or a few steroids, but are more likely to be detected by profiling. Profile analyses must be expected to add to the understanding of metabolic abnormalities and diseases.

Analysis of metabolic profiles was introduced approximately seven years ago for urinary steroids, sugars, sugar alcohols, aromatic acids, plasma sugars and serum acids of the Krebs cycle [47]. Especially after the introduction of glass capillary columns, steroid profiles were studied with respect to establishing normal profiles and aberrations due to disease or stress [6, 7, 11, 18, 19, 48, 49].

Multicomponent profile analysis with special emphasis on organic urinary acids and the detection of inborn errors of metabolism, has been developed to a very advanced state by workers in Oslo [50—54]. Together with other

workers investigating abnormalities in urinary acids [25, 55], more than twenty new inborn errors of metabolism were discovered by GC or GC—MS, many of which were caused by enzyme deficiencies in amino acid metabolism. In addition to new disorders, substantial new information was obtained about metabolism in known defects.

A broad study on abnormal organic acidurias in mentally retarded patients was described by Watts et al. [56]. From 1778 mentally retarded patients, 5% had an abnormal organic aciduria. As expected, the most frequently observed abnormalities were those of phenylalanine metabolites in cases of phenylketonuria. An excellent review on profiles of organic acids has been given by Jellum [57].

Low-molecular-weight and volatile metabolites in blood [35, 36, 58—60] and urine [32—34, 61—65], and to a lesser extent also in cerebrospinal fluid [61], breath, saliva and tissue, have been profiled with the object of establishing normal patterns, on the basis of which pathological abnormalities can be recognized. The topic has been reviewed by Politzer et al. [66].

Detailed studies have been made in our laboratory on the volatile compounds in urine of patients with diabetes mellitus. In comparison with normal individuals, diabetic patients develop distinct changes in the ketone and alcohol excretion [63]. High total 4-heptanone (4-heptanone plus its precursor, a β-ketocarboxylic acid) was found in urine of approximately 75% of the diabetic patients with balanced glucose levels [65]. During hypoglycemic periods the total 4-heptanone excretion increases, in severe hyperglycemic periods it decreases. Increased ethanol excretion is found in 80% of the urines, whereas higher-molecular-weight alcohols were detected mainly in the urine of patients with diabetic complications.

4. MASS FRAGMENTOGRAPHIC DETERMINATIONS AND REFERENCE METHODS

4.1. Direct mass fragmentography

Selective ion monitoring was first introduced by Sweeley et al. [67] and by Hammar et al. [68], who applied the technique to the identification of chlorpromazine and its metabolites in human blood and called it MF. Since then the potentials of this highly sensitive and specific method have been used for qualitative detection and quantitative assays. The detection limits are in the low picogram range (e.g. 4-heptanone, 2—5 pg). In order to interfere in an assay, a compound must have the same GC retention behaviour and the same specific ions, which is not very likely to occur. In quantitative analyses, calculations are based on calibration curves. To correct for losses in the sample preparation procedure as well as for uncontrolled variation of the instrumental conditions, internal standards should be used from the beginning of the procedure. They are either compounds with similar chemical properties, isomers or homologous substances, or substances labeled with stable or radioactive isotopes (isotope dilution method).

We have chosen the isomer 3-heptanone for MF determination of total 4-heptanone by SID using molecular ion *m/e* 114 [36, 69]. Very low background noise is observed for this ion, and the determination can be performed in a

non-concentrated extract obtained by a single-step extraction of urine with cyclohexane. The calibration curve is linear over more than three orders of magnitude [69]. The excretion of total 4-heptanone in normals ranges between 50 and 450 μg/24 h, in diabetics it is increased up to several mg/24 h. Using overlapping injections, the analysis time is approximately 8 min.

One of the obstacles for a wide use of GC—MS in clinical chemical routine laboratories is the fact that sample preparation prior to GC—MS analysis is often laborious. In general, aqueous samples should not be introduced into the mass spectrometer. For the MF determination of ethanol in urine and serum of diabetic patients we have overcome this problem by by-passing the water between the outlet of the GC-column and the interface to the mass spectrometer. In this way we analyze ethanol in a true micromethod by directly injecting 1 μl of urine or serum (Fig. 4).

A large number of publications has appeared on MF determination of biogenic amines and their metabolites in urine, plasma, cerebrospinal fluid or brain tissue. Some of the authors use internal standards of type 1 [70—73], others deuterium-labeled species [74—77].

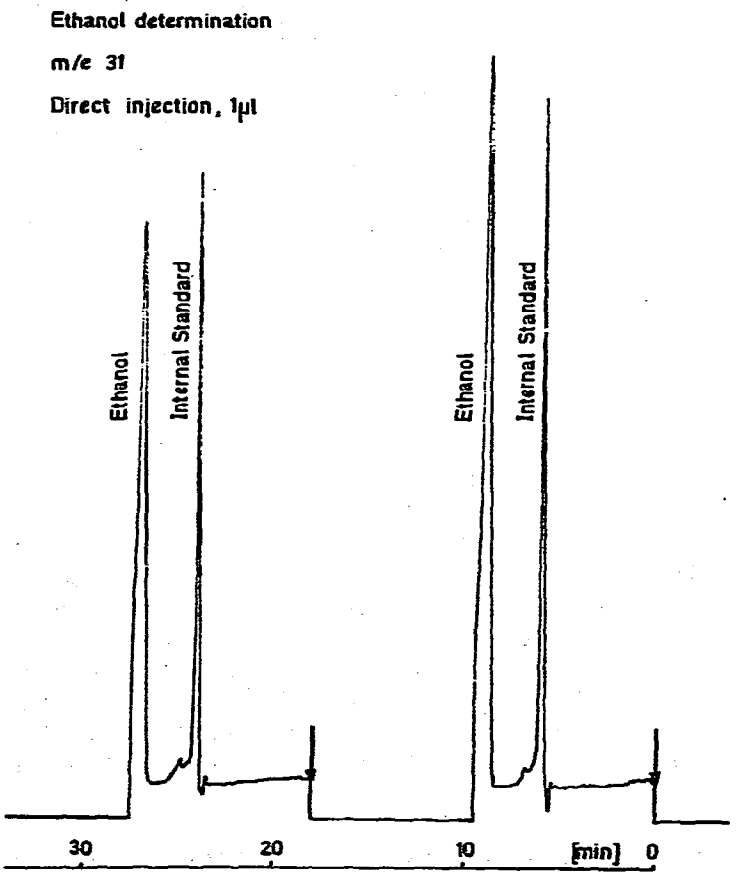

Fig. 4. MF determination of ethanol in urine. SID with *m/e* 31, $H_2C = \overset{+}{O}H$, direct injection of 1 μl of urine. Internal standard: diethyl ether.

Direct MF for steroids was used very early by Siekmann et al [78] and by Adlercreutz and Hunneman [79].

Using MF together with isotope dilution, reference and definitive methods were established for clinical chemistry. The MS determination of calcium with ^{44}Ca as the added isotope [80] gives results within a few tenths of one per cent of the true or absolute value.

For substrates and steroids, MF methods with isotope dilution have been developed mainly by workers led by Breuer and Björkhem, respectively. Methods are available for steroid hormones such as oestrogens [81, 82, 90], testosterone [81, 82, 84—87], 5α-dihydrotestosterone [84], progesterone [89], aldosterone [81—83] and cortisol [81, 82, 88], for cholesterol [91, 92], triglycerides [93], urea [94] and for glucose [95]. Deuterium-, tritium- and ^{14}C-labeled internal standards were used for the steroids, deuterated glycerol trioleate and glucose for the triglyceride and glucose determinations, respectively, and ^{15}N-labelling for urea. Mass differences between unlabeled and labeled compound should be small, otherwise partial chromatographic separation may occur. Variation coefficients were mostly between 1.3 and 4% for the various parameters, whereby pipetting errors are perhaps the most important source of variation. Special attention was also directed toward the type of derivative, heptafluorobutyric esters giving higher sensitivity than trimethylsilyl ethers.

Correlations between MF and radioimmunological or chemical methods showed either good agreement or lower values measured by MF (progesterone, cortisol), most probably as a result of the higher specificity of MF. The use of MF with isotope dilution has been reviewed by Björkhem et al. [96].

Using the advantage of a quadrupole mass spectrometer to simultaneously detect many specific ions over a broad *m/e* range, twelve amino acids from 50 μl of plasma or urine were determined with deuterated standard amino acids [97].

4.2. Computerized mass fragmentography

Whereas in direct MF one specific ion, or a small number of specific ions, has to be pre-selected prior to analysis, computerized MF allows the use of any recorded ion after the GC—MS run for specific detection or determination of a substance. This is especially suited to profile analyses. However, the advantage of the greater flexibility of this method must be weighed against the disadvantage of considerably lower sensitivity, because a larger mass range is scanned and fewer ions of one type reach the multiplier. If not prohibited by too low sample concentration, the method has a very wide use. From complex profiles, selective patterns can be obtained by computerized MF, giving information on the presence of a single compound or groups of compounds [34, 36]. Computer MF has proved very valuable for steroid [9, 12, 13, 98], amino acid [99] and bile acid [100, 101] analyses.

5. IN VIVO EXPERIMENTS WITH SUBSTANCES LABELED WITH STABLE ISOTOPES

With the increased use of MS in clinical laboratories it became possible to

use stable isotope labeling for in vivo experiments and diagnosis, and to complement or replace experiments with radioactive tracers, thus eliminating possible radiation hazards connected with radioactive substances. Compounds labeled with stable isotopes can be easily detected and quantitated in blood, urine or tissue by MS and MF.

By administering deuterium-labeled homovanillic acid (HVA), the turnover of HVA in man was determined [102]. Very detailed studies on phenylalanine and tyrosine metabolism were published by Curtius and coworkers. Patients with phenylketonuria and hyperphenylalaninemia were loaded with deuterated phenylalanine and the aromatic acids were determined in urine. No tyrosine metabolites were found [103], expressing a deficiency in the enzyme phenylalanine-hydroxylase. Administration of deuterated L-tyrosine to patients with phenylketonuria and determination of the excretion of Dopa metabolites, suggested that the tyrosine-3-hydroxylase activity and the formation of catecholamines depend on the phenylalanine concentrated in plasma [104].

Deuterated ethanol has been extensively used by Cronholm et al. in studies of biosynthetic pathways, e.g. the biosynthesis of cholesterol and bile acids [105] or Krebs cycle acids [106].

6. CONCLUSION

Since MS, especially in the form of GC—MS, is the most flexible and most powerful analytical technique available today for organic substances that are volatile or can be made volatile, and that are present in complex mixtures of biological origin, it is indispensable for biochemical and clinical research. Its potentials would be enhanced even more by combination with other techniques, particularly high-pressure liquid chromatography, thus eliminating to a large extent the requirement of volatility.

A mass spectrometer is no longer the formidable instrument, if it is operated by well trained personnel. However, because of its great potential a GC—MS system should not be hindered by analyses that can be performed by simpler and more economical methods. Only when the separating power, the sensitivity and the specificity of GC—MS are needed or when no other method is available for the analysis, GC—MS should be used. Often GC alone will suffice.

Instrument manufacturers are beginning, and should be encouraged to continue, to develop smaller, more economical, less flexible instrumentation, which still offers the optimal efficiency for a given type of analysis. In clinical chemistry, today's mass spectrometers are used in the first place in research and in diagnostic centers specializing in detailed metabolic investigation for diagnostic purposes.

7. SUMMARY

In clinical chemistry and clinical biochemistry mass spectrometry is used mainly in combination with gas chromatography, in some cases supported by a computer. The combination is distinguished by its separating efficiency for complex mixtures of substances and by its high sensitivity together with very

high specificity. An amount of 10—100 ng is sufficient for the identification of an unknown substance, and even 1—100 pg can be adequate for qualitative detection or quantitative determination by mass fragmentography. The principal areas of application are: (a) structural identification of substances; (b) profile analyses and pattern recognition; (c) mass fragmentographic determinations and reference methods; (d) in vivo experiments with substances labeled with stable isotopes.

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