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REVIEW

PROFILING OF HUMAN BODY FLUIDS IN HEALTHY AND DISEASED STATES USING GAS CHROMATOGRAPHY AND MASS SPECTROMETRY, WITH SPECIAL REFERENCE TO ORGANIC ACIDS

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1. INTRODUCTION

One of the trends in modern biomedicine is the increasing understanding that many, if not all, diseases may be linked in some way with deviations from, or alterations in, one or more of the several thousand chemical reactions that normally take place inside the cells and body. It does not seem unreasonable to assume that if one were able to identify and determine the concentrations of all compounds inside the human body, including both high- and low-molecular-weight substances, one would probably find that almost every known disease would result in characteristic changes in the biochemical composition of the cells and of the body fluids. Such a "total" analysis is, of course, impossible to carry out at present. However, the promising results achieved during the last few years have substantiated the importance of multicomponent analyses. Chromatographic profiling of the body fluids in particular has been useful both for diagnostic purposes and for obtaining new information about the biochemical reactions that take place inside the body in healthy and diseased states.

Most workers have favoured the use of gas chromatography (GC) for separation purposes, combined with mass spectrometry (MS) to identify the individual components in the body fluids. It is, however, important to bear in mind that GC and GC—MS can handle only volatile constituents and compounds that can be converted into volatile derivatives. Estimates have indicated that, because of this limitation, gas-phase analytical methods can only detect about 20% of the total number of substances present in complex biological materials [1,2]. The needs for alternative analytical methods are therefore obvious. High-performance liquid chromatography (HPLC) is considered to be one such alternative [3—6], although its methodology has not yet been

advanced to the stage where, e.g., mixtures of hundreds of different proteins, lipids, polysaccharides, nucleic acids, etc., can be separated. Recent advances, however, such as the computer-operated multi-wavelength array spectrometer detector [7,8], the coupling of HPLC to mass spectrometers [9—11] and new areas of application [12—14], indicate that HPLC will find its place beside GC—MS, where it is badly needed for multi-component analyses of high-molecular-weight and/or temperature-labile compounds. Isotachophoresis [15] and high-performance thin-layer chromatography (HPTLC) [16] may also become of considerable value in the analysis of body fluids in the future, as these techniques also have the potential of determining many of the widely different substances present in biological materials. Rapid-scanning fluorescence spectroscopy [17] and ion chromatography [18] are two recent techniques that might prove to be useful for the analysis of biological materials.

At present, however, it is clear that GC, and in particular GC—MS, are the major techniques for multi-compound analyses of the body fluids. Therefore, only these gas-phase methods will be considered here. The profiling approach is applicable to many different classes of biological compounds, from volatiles to complex carbohydrates, and several reviews have appeared recently. Volatiles were reviewed by Politzer et al. [19] and it is striking that the methodology has now been advanced to the stage where only 50—100 μl of body fluid is required for a complete recording of all of the volatiles present [20]. The scope of MS in clinical chemistry was reviewed by Roboz [2] and by Lawson [21], and the profiling approach in the study of microorganisms is considered in a recent book edited by Mitruka [22]. Comprehensive reviews on GC [23] and MS [24] discuss instrumentation, applications and experimental results.

The analyses of amino acids by paper (PC) and ion-exchange chromatography have proved over the last 25 years to be of great clinical value. In a similar manner, chromatographic profiling of organic acids in particular has become of diagnostic importance and has led to an improved understanding of many human disorders. It is therefore the purpose of this paper to review recent advances in organic acid profiling of human body fluids in healthy and diseased states.

2. RECENT ADVANCES IN INSTRUMENTATION PERTINENT TO CHROMATOGRAPHIC PROFILING

A. GC and combined GC—computer

Many modern GC instruments, available from over 500 manufacturers [23], are suitable for analyses of the widely different, often labile constituents of body fluids. All-glass systems seem to be preferred for this type of separation and packed GC columns with some type of silicone oil as the stationary phase (e.g., SE-30, OV-17, OV-22) have so far predominated. However, when the purchase of a new instrument is considered, many workers in the field now agree that the instrument of choice should be designed to handle glass capillary columns also, as these columns are becoming increasingly useful for profiling work. Excellent thermostable, wall-coated open-tubular glass capillary columns

suitable for the analysis of body fluids are now commercially available from several manufacturing companies. The glass capillary column not only offers greatly improved separation, but as the peaks are very sharp and as adsorption on the column wall is negligible, increased sensitivity is actually obtained. However, as was proposed in a round-table discussion on biomedical applications of GC—MS held during the 7th International Conference on Mass Spectrometry in Florence in 1976 [25], one should not advocate the view that packed columns are obsolete for the analysis of body fluids. Indeed, many problems, e.g., routine analyses of a given drug or of certain metabolites, can be carried out equally well on packed columns. Recent results (e.g., refs. 26—31), on the other hand, show that when one is dealing with *multi-component* analyses, where the aim is to separate and identify as many constituents in the body fluids as possible, then capillary columns are superior and give considerably more information.

Computerized GC has undergone major developments during the last 2—3 years. On-line mini- and micro-computer systems, ranging from programmable calculators to advanced time-shared systems, now offer the analyst a number of possibilities, such as adaptive program interaction, integrator/calculator functionality, instrument automation, automatic data acquisition and data reduction, real-time control of the sampling system, digital flow and temperature controllers, automatic peak detection, automatic baseline correction, calculation of retention indices, prediction of component resolution, detection and measurement of overlapping peaks and pattern recognition techniques (for references, see ref. 23). For example, a fully automated high-resolution GC system suitable for chromatographic profiling of the volatile constituents in body fluids was recently described [30]. It incorporates repetitive sampling on to a glass capillary column and simultaneous flame-ionization and nitrogen-sensitive detection prior to data acquisition and computer handling. Other sophisticated, automated and computerized GC systems have also been designed for studying volatiles (e.g., ref. 31) and for studying plasma lipid profiles [32]. Computerized GC has also proved useful for the chromatographic profiling of microorganisms [22].

Although automated high-resolution GC methods combined with powerful computer evaluation techniques are likely to find an increasing number of applications in the biomedical field, these methods cannot provide positive identification of individual GC peaks. Retention times alone are unfortunately not sufficient for identification purposes and should be used with caution when one is dealing with biological fluids. These materials, particularly urine, are so complex that even when highly efficient capillary columns with nearly 100,000 theoretical plates are used, many, if not most, of the GC peaks are still not resolved, but consist of a mixture of two or even more metabolites [25]. Much of the diagnostic value of chromatographic profiling ultimately depends on the identification of "disease-specific" compounds and it is therefore clear that methods in addition to GC must be sought. It is fully recognized to-day that MS at present is the best choice for providing such information.

B. Combined GC—MS—computer

Excellent reviews on MS and on GC—MS—computer methods have been written, e.g., by Burlingame et al. [24, 33], Brooks and Middleditch [34], Horning et al. [35], Roboz [2], Lawson [21] and Politzer et al. [19] and, in addition, several books (e.g., refs. 22, 36—39) are available.

Both electron-impact ionization and chemical ionization techniques are now widely used. The availability of mini- and more recently micro-computers with suitable disc-oriented operating systems, graphic display units and electrostatic printer/plotter units as well as the continually increasing performance/cost ratios have greatly improved and facilitated the technique of MS [24]. Even the physical sizes of the equipment have changed recently, and a complete GC—MS system that can be placed on a laboratory bench-top is now commercially available.

It is now possible, both with quadrupole instruments and with the newer magnetic sector mass spectrometers, to carry out the fast scanning (about 1 sec) required for tandem operation with capillary columns, making the use of these high-resolution columns even more attractive. Selected ion monitoring (SIM), also called mass fragmentography and multiple-ion detection, has become completely computerized in recent years, i.e., software-operated in contrast with the traditional hardware-based systems (e.g., ref. 40). The high sensitivity and specificity of SIM is well recognized and this technique is now widely used for qualitative and particularly for quantitative analyses of a variety of endogenous metabolites and of exogenous compounds, e.g., drugs and drug metabolites, that occur in physiological fluids (for example, see the review by Falkner et al. [41] and the books cited in refs. 36—39, 42 and 43).

Some novel techniques of interest for multi-compound analysis include elemental composition chromatography (accurate mass chromatography) [24, 44], field ionization MS combined with computer techniques [45] and ultramicro-scale automatic MS analyses [46]. In the first of these methods, glass support-coated open tubular columns have been coupled to a high-resolution (dynamic resolution about 10,000) mass spectrometer—computer system. The scan cycle time is about 10 sec for the mass range 60—800 and several hundred high-resolution mass spectra can be obtained during a chromatographic run [44]. In the second method [45], extracts of the biological material (e.g., urine) are introduced into the mass spectrometer without prior chromatographic separation. The instrument is fitted with a heatable field ionization source. This type of ionization results in minimal fragmentation of the components in the mixture and therefore produces molecular weight profiles of the analysed samples. Using suitable computational methods, the system could readily differentiate between normal urine and urine from patients with infectious hepatitis [45].

The third system utilizes a mass spectrometer, coupled to automatic sample preparation devices and an electro-optical ion detector/computer system capable of detecting many ion species simultaneously [46].

Some recent advances in pyrolysis MS [47] are of interest. Thus, it is now possible to obtain reasonably reproducible "fingerprints" of complex biological samples such as whole cells, using either the Curie-point principle or the carbon dioxide laser beam for flash heating of the samples. This method is already

of value for characterizing multi-compound mixtures such as bacteria [22, 47].

During the last few years, much attention has been focused on the problem of interfacing liquid chromatography (LC) with MS. Several different systems are now in use, e.g., the method developed in Horning's laboratory [9], where a portion of the vaporized LC solvent is forced through an atmospheric-pressure ionization source [48]. In the system used by McLafferty et al. [10], about 1% of the LC effluent is introduced into the ion source of a chemical-ionization mass spectrometer. The vaporized solvent serves as the reagent gas for the sample. A third system utilizes a moving-wire or, more recently, a moving-belt interface. The set-up consists of an auxiliary vacuum chamber through which the wire or belt continually carries aliquots of the LC column effluent, and where the solvent is removed. The sample is further transported into the ion source of a quadrupole instrument where the final vaporization and ionization take place (e.g., ref. 11).

C. Library search of mass spectra and computer evaluation of chromatographic profiles

Apart from pattern recognition techniques that have been of great value in, for example, analyses of volatiles (see p. 430) and for bacterial classification [22], most of the work on profiling ultimately ends up with the problem of identifying certain peaks on the gas chromatogram. Manual interpretation of the corresponding mass spectra is often difficult and time consuming. The visual comparison of unknown spectra with a catalogue of known mass spectra often gives valuable clues with regard to the structure. The collection entitled "Mass Spectra of Compounds of Biological Interest", compiled by Markey et al. [49] and available through the National Technical Information Service, U.S. Department of Commerce, Springfield, Va. 22161, U.S.A., is very useful. The "Registry of Mass Spectral Data" [50], containing about 25,000 spectra, and the "Eight Peak Index" [51], which contains over 31,000 entries, are also valuable although most of the spectra in these collections relate to non-biological compounds. Many sophisticated computer programs, including interactive library searching, heuristic techniques and learning machine techniques, have been published [24, 52, 53]. At least two such search programs are now available over international computer networks. One system, McLafferty et al.'s self-training, interpretative and retrieval system for mass spectra (STIRS) [54], including probability based matching [55], is available through TYMNET [56]. The second system, developed by Heller at al. [57], is available through the General Electrics computer network, and in Scandinavia is available through SCANNET [58]. Several systems [59, 60] are now in operation for the rapid computerized identification of compounds in complex biological mixtures by GC—MS. The system used in Biemann's laboratory has been widely used for drug analyses [61] and the system developed by Sweedy et al. [60] has been used, for example, for the rapid characterization of urinary organic acids. Sjövall's group (e.g., ref. 62) has been used advanced computer systems for several years for the interpretation of steroid profiles. The system of Jellum et al. [63] has been designed to recognize anomalies in multi-compound mixtures, and is in principle able to detect the presence of abnormal com-

pounds as well as the lack of normal constituents. Although the first version of the program needs refinements, the approach has already proved useful [64].

In many instances the need for computer evaluation of the chromatographic profiles may seem unnecessary. This may be true when one is dealing with the diagnosis and detection of metabolic disorders that lead to gross metabolic changes. Other clinical conditions, however, may result in more subtle, yet perhaps important, changes in the metabolic patterns. It seems reasonable to assume that the best way to detect these minor alterations will be by means of advanced computer methods. For further discussion on computers and MS, see refs. 24, 52 and 53.

3. TREATMENT OF THE HUMAN BODY FLUIDS PRIOR TO CHROMATOGRAPHIC SEPARATION

A. Collection, storage and transport of specimens

Although increasing in number, there are still few clinical laboratories and hospitals that have at their disposal combined GC—MS—computer equipment. Many more laboratories, however, are equipped with simple gas chromatographs, and much useful screening of body fluids for abnormal metabolites can be carried out by GC alone. However, when a patient presents with an abnormal GC peak, the structure and identity of the substance concerned normally needs to be verified by MS and the sample can be submitted to a specialized laboratory. Transport is also necessary when clinicians have to rely on specialized laboratories for carrying out the complete chromatographic profiling. It is, of course, always important that the samples should undergo as little change as possible from the time of collection until they reach the place where the multi-component analyses are to be carried out. Recommendations for collection, storage and transport of physiological fluids for chromatographic profiling have been made [65]. Blood samples from fasting patients and urine collected in the morning appear to be preferred, and no preservatives should be added. The urine and serum samples should be frozen and stored at -20°C or preferably at an even lower temperature so as to prevent decomposition of certain metabolites, e.g., peptides [see 25]. When morning urine samples are analysed, quantitative results are usually expressed relative to the creatinine content. For more exact quantitative data, 24-h urine samples should be collected. Care must be taken to obtain information about drug intake and particular dietary habits [66—69]. Whenever unusual peaks are noted on gas chromatograms, especially in laboratories that lack GC—MS—computer facilities, it is a good rule to suspect the peaks of being drugs or drug metabolites [65]. This suspicion can sometimes be confirmed either by repeating the fluid collection from the patient at a later date when drug therapy has been discontinued, or by obtaining samples of physiological fluids from suitable control subjects who are receiving the same drugs as the patient. One should also realize, however, that many metabolic disorders are of intermittent types [70].

B. Isolation of organic acids from urine

Three principal methods for the isolation of the acidic constituents of urine prior to derivatization and GC are currently in use. The first is based on solvent extraction, usually with diethyl ether and/or ethyl acetate. Batchwise extractions using manual or mechanical shaking are normally carried out, although these procedures are not quantitative and polar compounds such as di- and tricarboxylic acids and polyhydroxy acids are poorly extracted [71]. More quantitative extraction is obtained by continuous extraction overnight, e.g., in a Soxhlet apparatus. Improved extraction is also obtained by saturating the sample with salts, e.g., sodium chloride before extraction. This treatment, however, precludes the use of the remaining aqueous phase for further analyses of, e.g., carbohydrates, amino acids and conjugates [1].

A good solvent should have a high volatility, a high solvent power with low water solubility and a high stability, and it should be commercially available in a high-purity analytical or chromatographic grade. In a recent investigation [72], isopropyl chloride was proposed as the solvent of choice for the extraction of volatile compounds in biological fluids. From the physicochemical characteristics (b.p. 35.7°, dielectric constant 9.82 (diethyl ether 4.3) and water solubility 1.3% (diethyl ether 6%), it appears that isopropyl cloride should also be suitable for the extraction of organic acids.

The second method for the isolation of the acidic constituents is based on anion exchange, as first suggested by Horning and Horning [73] and since used in many laboratories. Ion-exchange methods give quantitative recoveries of the organic acids in urine. Comparative studies on solvent-extraction and ion-exchange procedures have recently been carried out [71]. DEAE-Sephadex appears to be the most widely used ion-exchange material, although other resins, e.g., Dowex 3, are also suitable [74]. After capture on the resin, the acids are usually eluted with aqueous pyridinium acetate (e.g., refs. 66, 73) before lyophilization. In order to avoid any loss of the more volatile acids during the lyophilization step, it has been suggested that the column should be eluted with hydrochloric acid followed by neutralization of the eluate with sodium hydrogen carbonate, before lyophilization [75]. The direct silylation of the resulting sodium salts then follows [75, 76]. Thompson and Markey [71] introduced a clean-up step prior to ion exchange on DEAE-Sephadex. Their method involves removal of the interfering inorganic sulphate and phosphate as insoluble barium salts, followed by oxime formation, ion exchange, silylation and GC. Lawson et al. [66], however, warned that many organic acids are coprecipitated in this method, and that several organic acids would decompose when the alkaline barium hydroxide is added.

A third, more special, method, limited to the isolation of the more volatile constituents, makes use of either steam distillation [77—79] or vacuum distillation [80] to isolate the organic acids from the biological materials.

The problem of choosing between the solvent-extraction and anion-exchange methods often arises. It has been the experience of several groups that if the problem concerns screening for gross metabolic disorders, the detection of which does not require quantitative separation procedures, then solvent extraction is applicable. Even metabolic disorders that result in the excretion of

polar compounds such as pyroglutamic acid [81] or glutaric acid [82] are easily detected by solvent-extraction methods. On the other hand, very polar and highly water-soluble compounds such as tetronic and deoxytetronic acids are poorly extracted into organic solvents and may remain undetected unless ion-exchange methods are used. For quantitative work (e.g., refs. 66—68) and work involving subtle changes in the chromatographic profiles, the use of anion-exchange procedures is not only recommended, but is essential. Some laboratories therefore prefer always to use the quantitative methods, whereas others, particularly those engaged in diagnosis and studies of metabolic disorders, have found it convenient to be able to use both isolation procedures. Solvent extraction is used in emergency cases, e.g., on samples from severely ill acidotic children, in order to obtain a qualitative answer as soon as possible. Anion-exchange methods, which are more laborious and time consuming, are subsequently used for quantitative extraction and assay of the pathological metabolites.

C. Isolation of organic acids from protein-containing fluids

Urine is the only human body fluid that normally is devoid of proteins. All other body fluids (and tissue homogenates) contain small (in amniotic fluid, spinal fluid) or large amounts of protein (in serum, seminal fluid, synovial fluid, etc.). Urine, spinal fluid [83] and amniotic fluid [84] can therefore be extracted directly as discussed above, whereas most other physiological samples usually are handled so as to avoid interference by the proteins. If non-destructive methods (gel filtration, dialysis, membrane filtration) are used to remove the proteins prior to the GC analyses, the tightly protein-bound and/or water-insoluble low-molecular-weight substances (e.g., long-chain fatty acids) will be lost. Several organic acids have a tendency to become more or less protein bound, indicating that removal of protein by non-denaturing methods should be used with caution if the aim is to obtain a total organic acid profile. Other classes of metabolites, e.g., carbohydrates and amino acids, are not protein bound, and membrane filtration through a Millipore PSAC filter, for example, yields a protein-free eluate that is ideally suited for, e.g., carbohydrate profiling by GC methods [29]. Protein precipitation with, e.g., sulphosalicylic acid followed by extraction of the organic acids by organic solvents is sometimes used, although certain organic acids may be coprecipitated with the denatured protein. Direct extraction of serum, e.g., with the ion—solvent pair ammonium carbonate—ethyl acetate, is widely used for the extraction of drugs, but this method obviously does not extract the organic acids.

A convenient method for obtaining profiles of total organic acids in protein-containing body fluids and tissue homogenates makes use of ethanol as a precipitating agent (e.g., refs. 1, 2, 85 and 86). After removal of the proteins by centrifugation and removal of the ethanol in vacuo, the resulting aqueous solution can be treated as if it were urine.

Fatty acids and lipids are usually extracted by special methods, e.g., Folch extraction. The literature on the analyses of these compounds in biological fluids is vast. Procedures for their extraction and analysis by GC and GC—MS

methods can be found, for example, in three issues of Journal of Chromatographic Science (Sept. 1975, Oct. 1975 and Jan. 1976) devoted to this topic. Packed GC columns containing liquid phases such as DEGS, DEGA, EGSS-X, Silar 5C and 10C or Apolar 10C, are used extensively for the separation of fatty acids, usually as methyl esters. Methods for the separation of underivatized fatty acids have also been published [87, 88]. Highly efficient glass capillary columns are used to separate *cis* and *trans* isomers [89]. A review on the analysis of lipids in general has recently appeared [90].

4. DERIVATIZATION OF THE ORGANIC ACIDS

The "organic acid fraction" of biological fluids contains mono- and polycarboxylic acids, mono- and polyhydroxy acids, keto acids, phenols, phenolic acids and conjugates of the organic acids, particularly with glycine. Numerous publications have dealt with methods for preparing volatile derivatives of the different organic acids and the most widely used approches are discussed below.

A. Silylation, oximation

Trimethylsilyl (TMS) ethers and esters are probably the most popular derivatives used for studying the organic acids (e.g., ref. 85). TMS derivatives are comparatively easy to prepare, safe to handle and most of them have excellent chromatographic properties. Further, deuterium-labelled TMS derivatives can readily be prepared with commercially available silylating reagents. The latter approach is frequently of great value in elucidating the structures of unknown metabolites by GC—MS. The collections of mass spectral data (see p. 432) contain more information on TMS derivatives than on any other type of derivative. The most widely used silylating reagents are bis(trimethylsilyl)trifluoroacetamide (BSTFA) and bis(trimethylsilyl)acetamide (BSA) (with or without pyridine, trimethylchlorosilane or other catalysts), which form derivatives with the carboxyl groups, hydroxyl groups and phenol groups of organic acids. Sodium salts are generally more difficult to silylate than the free acids, although recent investigations [75, 76] have shown that BSA in the presence of trimethylchlorosilane and/or hydroxylamine converts the sodium salts directly into volatile TMS derivatives of the organic acids. Certain metabolites, particularly keto acids, have a tendency to yield multiple derivatives, and there are problems also with the silylation of short-chain dicarboxylic acids [74] and with *o*-hydroxyhippuric acid (salicyluric acid [91]). In both instances the corresponding methyl esters appear to be better. Previously it was observed that N-acylglycines also gave multiple peaks after trimethylsilylation, but this effect can now be avoided [92]. The GC behaviour of 20 N-acylglycines of clinical interest is described in refs. 92 and 93. Keto acids are more readily determined if double derivatives are prepared, particularly oxime—TMS [94], methoxime—TMS [95] and ethoxime—TMS derivatives [66]. Other O-substituted oxime—TMS derivatives, e.g., benzyloximes, have also been considered by Lawson et al. [96] although they prefer ethoxime—TMS derivatives for the determination of urinary organic acids in general [66—68]. Alipathic 2-keto acids can also be determined with o-phenylenediamine and BSTFA, yielding

O-TMS—quinoxalinol derivatives that are suitable for GC analysis [97]. The separation of the many biologically occurring keto acids is best acieved by using highly efficient glass capillary columns [98]. Combined silylation and methylation also yields derivatives with excellent chromatographic properties, and this procedure has been successfully for many years by Horning and co-workers (e.g. refs. 35 and 95).

Combined silylation and ethylation has been the preferred derivatization method in studies on the simultaneous determination by selected ion monitoring of the levels of four acid metabolites of catecholamines in urine, serum and cerebrospinal fluid [99].

B. Methylation

The methyl esters of organic acids also have excellent chromatographic properties and can readily be prepared. The mass spectra of methyl esters are usually simple, and are often easier to interpret than those of the corresponding TMS derivatives. Usually it is therefore easier to predict the fragmentation pattern of a methyl ester than that of a silyl ester/ether. This is frequently very useful in profiling work, because on the basis of a predicted mass spectrum one is often able, using mass chromatography, to search for the presence in a body fluid of a given metabolite, even if the authentic compound and/or information on its GC—MS behaviour are lacking.

In several laboratories, therefore, (e.g., refs. 1, 85, 100—102) methylation techniques are frequently used. Despite the hazards associated with diazomethane, it still appears to be the preferred methylating agent, the method being smooth, simple and rapid. This method, like silylating procedures, may also lead to certain by-products, artifacts and unexpected results. For example, more than one derivative is usually formed by the action of diazomethane on keto acids; pyrazolines are formed as by-products during the methylation of fumaric and aconitic acids; and an artifact resembling 3-hydroxypropionic acid is formed if water is present during the methylation [104]. Diazomethane reacts not only with carboxyl groups, but also methylates the hydroxyl groups of phenols and the thiol group of thiols. Thus, a number of important phenolic acids, e.g., *p*-hydroxyphenyllactic acid, may yield one or two derivatives depending on the temperature and length of treatment with diazomethane. For further discussion on this topic and on other artifacts and pitfalls associated with the chromatographic profiling of body fluids, see p. 439 and refs. 103, 104.

Alternative methylation procedures include esterification with methanol/hydrochloric acid, methanol/thionyl chloride or methanol/boron trifluoride and on-column methylation [105]. In the last method, the organic acids are converted into their trimethylanilinium salts by the addition of trimethylanilinium hydroxide, and subsequent pyrolysis of the salts in the injection port of the gas chromatograph generates the methyl esters [105, 106]. This method, combined with neopentylidene protection of the amino groups, is also suitable for the rapid derivatization and analysis of amino acids by GC [107].

Selective esterification of the carboxyl group can be achieved by the classical

method of treating the organic acids with alcohol in the presence of an acidic catalyst. Subsequent removal of excess of alcohol and acid before injection into the gas chromatograph often results in loss of the more volatile esters.

An alternative method for the selective derivatization of the carboxyl group involves addition of silver nitrate to an aqueous extract of the organic acids, followed by lyophilization and treatment of the dry silver salts with ethyl iodide (or methyl iodide) dissolved in pentane [108, 109]. The ethyl (or methyl) esters will be formed in the pentane solution, which can be injected directly on to, e.g., a capillary GC column.

The relative merits of the various approaches considered in this chapter are frequently discussed. There is no simple answer to the problem, as all methods, including solvent extraction, ion exchange, trimethylsilylation and methylation. have their advantages and disadvantages. Most workers, however, will agree with what Stokke [110] pointed out in a recent editorial, namely that the main point is to become familiar with one method, and to learn its pitfalls and to be able to use alternative procedures when required.

5. SPECIAL METHODS FOR SHORT-CHAIN VOLATILE ACIDS

Short-chain (C_2—C_8) monocarboxylic acids are volatile and therefore require no derivatization. Special methods for handling these acids, several of which are associated with metabolic disorders, have been published. In one of these [77—79] the volatile acids are separated after steam distillation (on a column containing neopentyl glycol adipate—orthophosphoric acid), or after vacuum distillation [80]. Other methods (e.g., refs. 111—113) utilize solvent extraction followed by separation on columns containing, e.g., Porapak [111] or FFAP (Carbowax 20M—nitroterephthalic acid [113]). In another method [114], the urine is mixed with Dowex 50-H^+ and the free acids released are separated on the porous polymer Chromosorb 105. In the method of Remesy and Demigne [115], as used by Wysocki et al. [116], the acids are separated on a column packed with 10% SP-1200 containing 1% of orthophosphoric acid on Chromosorb W. Special GC methods have been devised for the determination of acetate [117] and for propionic and methylmalonic acids [118].

The GC determination of the latter two acids in the presence of each other requires special care, as a portion of the free methylmalonic acid may become decarboxylated to yield propionic acid in the injection port of the gas chromatograph (e.g., ref. 119). The finding of large amounts of propionic acid in, e.g., the urine of patients with methylmalonic aciduria, may therfore be misleading. Possibly the simplest means of overcoming this problem is to use two different temperatures on the inlet block of the gas chromatograph, as suggested by Frenkel and Kitchens [118]. Their method is based on the spontaneous and quantitative decarboxylation of methylmalonic acid at 225° to give propionic acid. By utilizing another substituted malonic acid (ethylmalonic acid) as an internal standard, accurate quantitation is possible. Endogenous propionic acid is then measured at 130°, a temperature at which methylmalonic acid does not decarboxylate [118]. The use of ethylmalonic acid as an internal standard may be questioned, as it has been described as a normal con-

stituent of human urine (although present in small amounts) [120] and may occur in considerable amounts in, for example, patients with Jamaican vomiting sickness (p. 450) or with glutaric aciduria, type II (see Table 1). An alternative procedure was used by Stokke et al. [121] in their investigation of a patient with combined methylmalonic acidemia and β-hydroxy-*n*-valeric acidemia. Propionic and methylmalonic acids were first separated on buffered TLC plates so as to avoid loss of the volatile acids. The bands containing the two acids (as sodium salts) were subsequently analysed separately by GC (propionic acid as the free acid, methylmalonic acid as the methyl ester).

6. ARTIFACTS AND PITFALLS

The application of GC and GC—MS—computer methods to the analysis of body fluids is associated with many difficulties and pitfalls. There are problems of a technological nature (instrument breakdown), of a chemical nature (e.g., contaminated solvents, contamination from column bleeding and environmental contamination such as plasticizers), and problems related to the handling of the biological samples. Spiteller and Spiteller discussed several of these aspects in their book [122], which also contains the mass spectra of numerous commonly occurring contaminants. Plasticizers in biological materials have also been studied by other workers (e.g., refs. 123 and 124). Perry and Hansen [103] and more recently Jellum et al. [104] have reviewed the most usual pitfalls associated with the chromatographic profiling of biological materials using GC and GC—MS methods. Firstly, there are problems with collection and storage of the samples. Depending on the container used, contamination may occur from chemicals not only from plasticizers but also from rubber stoppers [104, 125], added preservatives and added anticoagulants [103, 104] (e.g., heparin contains benzyl alcohol as a stabiliser; see also ref. 126). Secondly, numerous problems exist with sample work-up and with the derivatization methods, e.g., formation of artifacts and multiple derivatives. The production of artifacts (e.g., crotonic acid formed from β-hydroxybutyric acid [127]) and the formation of unexpected compounds in the GC column (e.g., 5-hydroxycoumaran from homogentisic acid [104] and 3-methylcrotonic acid from 3-hydroxyvaleric acid [128]) may lead to serious errors. Decarboxylation reactions (e.g., of methylmalonic acid to yield propionic acid (p. 438) and of phenylpyruvic acid to yield phenylacetic acid [129]) during sample work-up and GC may lead to erroneous results. New compounds formed by trans-esterification processes [130] and artifacts produced by bacteria (e.g., benzoic acid [131, 132]) are potential sources of error. In methods where the biological material is made alkaline to prevent loss of volatile acids, one should realize that part of the glucose may be converted into acetate [133]. Perhaps the most serious problems arise from artifacts due to dietary factors and intake of drugs (e.g., refs. 103 and 104). Particular attention should be paid to drugs that are metabolized to give organic acids that one normally associates with metabolic disorders. An example is the anticonvulsant sodium dipropylacetate, which leads to increased urinary excretion of propionic acid [134], 2-oxodipropylacetic acid and 2-(*n*-propyl)glutaric

acid [135]. In general, many problems can be avoided if correct and complete information about drug intake always accompanies the samples submitted for chromatographic profiling. (See also a recent review on difficulties and pitfalls in the interpretation of screening tests for the detection of inborn errors of metabolism [136].)

7. NORMAL ORGANIC ACID PROFILES OF VARIOUS BODY FLUIDS

A. Normal patterns in urine from adults and newborns

A table of the urinary organic acids identified prior to 1973 was compiled by Markey et al. [137]. Since then, many new metabolites have been identified by GS—MS methods. More recent lists of the prevailing urinary acidic metabolites in normal adults [66, 138] and in newborns [139] have been reported. The list of Björkman et al. [139], showing the qualitative and quantitative pattern of organic acids in urine from newborns, also contains the characteristic mass spectral fragments of each compound. Comparison of the organic acids in adults and newborns shows that the latter urine contains large amounts of succinic, fumaric, 2-ketoglutaric and 3-hydroxy-3-methylglutaric acids. These acids are present in only small amounts in adults. Hippuric acid, on the other hand, is a minor component in urine from newborns and a major constituent of adult urine. Knights et al. [138], using glass capillary columns and MS, identified over 30 of the peaks in the chromatogram. They examined pooled urine specimens from controls and post-partum subjects; no major differences were observed.

B. Normal patterns in other body fluids and tissues

Most papers have dealt with urine, and less information exists on the organic acid composition of other body fluids. *Amniotic fluid*, at a mean gestational age of 17.6 weeks (range 15—20 weeks), contains lactate as the dominating water-soluble acid and smaller amounts of pyruvate, 3-hydroxy-butyrate, succinate, 2-oxoglutarate, citrate, 2-ketoisovalerate, 2-ketoisocaproate, malate and *p*-hydroxybenzoate [84]. Cholesterol [140] and fatty acids, the determination of which is important in connection with respiratory distress syndrome, are present (see p. 451). 2-Hydroxybutyrate is also found [141] in amniotic fluid, probably as a consequence of the high lactate content (see p. 445). The concentration of 2-hydroxybutyric acid in this fluid is about 100 times lower than that of the predominant acid, lactic acid.

Few normal organic acid profiles of *serum* have been published. In general, the fatty acids dominate whereas the water-soluble organic acids are present in small amounts owing to high renal clearance. Many organic acid profiles of sera from patients with metabolic disease have been reported (see Table 1).

Cerebrospinal fluid (CSF) contains, apart from the fatty acids (e.g., ref. 142), various other metabolites such as 3-methoxy-4-hydroxyphenylethanol, 5-hydroxyindoleacetate, citrate, homovanillate, 3,4-dihydroxyphenylacetate, 3,4-dihydroxyphenylethanol and 3-methoxy-4-hydroxyphenyl glycol [83]. Catecholamine metabolites are now frequently assayed in CSF by means of

selected ion monitoring with stable isotope-labelled compounds as internal standards. Several tryptophan metabolites in CSF have been determined by single-ion monitoring [143]. Lactate, succinate, citrate and maleate are normal constituents in CSF from newborns. Variations in the chromatographic profile of neutral and acidic metabolites in CSF from newborns and infants with different neurological disorders have been reported [144]. Further work, however, is required in order to determine whether the alterations seen are sufficiently specific to be used as major diagnostic criteria [144].

Capillary GC—MS methods have been utilized to evaluate the organic acid profile in *human saliva* [145]. Lactic, 2-hydroxyisocaproic, succinic and phenylacetic acids, 2,6-dibutylcresol (an antioxidant present in food), phenyllactic, *p*-hydroxyphenylacetic and *p*-hydroxyphenylpropionic acids, several fatty acids and cholesterol were identified in unstimulated saliva.

Seminal fluid also contains several organic acids of the type found in urine, with citric acid and fatty acids as dominating compounds (Jellum, unpublished results). *Synovial fluid* has been examined with both packed [146] and capillary columns [147].

Plasma from haemodialysis patients has been profiled with respect to volatiles [148]. *Dialysis fluid* from nephrectomized patients [149] contains organic acids of the same type as normally found in urine, plus additional compounds associated with uremia.

Little information on organic acid profiles of tissues exists, whereas certain other classes of compounds have been determined by GC methods. Thus, the volatile constituents of lung, brain and liver from rats have been assessed. There were marked differences, and it appears that certain volatile constituents may be characteristic of a particular tissue [150]. The volatiles that produce the odours of the human vagina [151], the amino acid composition of human nails [152] and the content of polyols and aldoses in cataractous human lens tissue [153] have recently been measured by GC and MS methods. Snedden and Parker [154] investigated the presence of altered purine metabolites in skeletal muscle of normal and gouty individuals before and after allopurinol therapy.

With the advent of glass capillary columns, increased sensitivity of the instruments and improved computer handling of the data, it is to be expected that the analysis of small (1—3 mg) tissue biopsies will become feasible and important in the future.

C. Identification of new normal metabolites

Lawson et al. [66] have identified some new polyhydroxy (aldonic and deoxyaldonic) acids in addition to those previously reported by Horning and Horning [73]. Thompson et al. [155] also reported on the occurrence of polyhydroxy compounds in urine and identified 4-deoxyerythronic, 4-deoxythreonic and 2-methylglyceric acids [155] as new normal metabolites. Fell et al. [156] determined 2-deoxytetronic acid in blood.

Many dicarboxylic acids have been identified as constituents of normal urine. Petterson and Stokke [157] found that a series of 3-methyl branched short-chain (C_4—C_8) dicarboxylic acids are excreted in small amounts, except

3-methyladipic acid, which often is present in considerable amounts (0.1—0.2 mmole per 24 h) in urine. Lindstedt et al. [158] have shown that 3,4-methylenehexanedioic acid (cyclopropaneadipic acid) is a normal urinary constituent (10 mg excreted per 24 h). They also identified an acetylenic compound (5-decynedioic acid) [159] and a series of *cis*- and *trans*-monounsaturated aliphatic dicarboxylic acids in normal urine [160]. Considerable amounts of cyclopropaneadipic acid have been found in urine after the intake of a certain cheese [104].

The presence of various furan-containing carboxylic acids in urine is well established. These metabolites appear to be of dietary origin [161]. Certain fructose-containing solutions designed for intravenous feeding may contain considerable amounts of furan derivatives, formed during heat-sterilization of the fructose solution. Patients receiving such mixtures will excrete large amounts of furan mono- and dicarboxylic acids in their urine [162].

Mamer and Tjoa identified 2-ethylhydracrylic acid in urine [163]. The compound stems from the degradation of isoleucine.

Duncan et al. [164] noted that in most of the GC profiles obtained on methylation of human urinary extracts, a peak, sometimes large and sometimes small, appeared immediately after hippuric acid (methyl ester). GC—MS and nuclear magnetic resonance (NMR) spectroscopy provided conclusive evidence that this peak was due to β-(*m*-hydroxyphenyl)hydracrylic acid. This compound was first identified in human urine in 1957 by two-dimensional paper chromatography [165] and is of dietary origin. Wadman et al. [166] have reported that β-(*p*-hydroxyphenyl)hydracrylic acid was present in the urine from a patient with gastrointestinal disease. The *para*-isomer is apparently not a normal urinary constituent.

Human urine also contains highly variable amounts (from non-detectable to a peak comparable in size to that of hippuric acid) of 4-hydroxycyclohexane-1-carboxylic acid of unknown origin [167]. The TMS derivative of this compound has a molecular weight of 288. An unknown compound with the same molecular weight was recently found in a patient with α-ketoadipic aciduria [168] and was tentatively suggested to be 1,2-butenedicarboxylic acid. As the retention times of this compound and of the newly recognized cyclohexanecarboxylic acid are similar, Bindel et al. [167] suggested that Przyrembel et al. [168] may in fact have isolated the cyclohexane compound from their patient.

Nearly all of the compounds mentioned above were separated on packed GC columns. A normal organic acid profile on such columns yields about 40—80 peaks. If the separation is carried out instead on modern glass capillary columns, at least three times as many peaks are seen [64]. Most of these peaks are still unidentified. In the future, the identification of a wide range of further metabolites should become possible.

D. Quantitative ranges and effects of individual variation and diet on the chromatographic profiles

Factors that influence the chemical composition of human body fluids have been the subject of numerous investigations in the last few decades. A

few years ago, Young and co-workers [169, 170] reported on the effects of a chemically defined diet on a wide range of urinary and serum metabolites. Witten et al. [171] also used a palatable standard diet and measured the means and standard deviations of the excretion rates of individual urinary organic acids, using GC—MS methods. In the latest and most comprehensive studies carried out by Chalmers and co-workers [66—68], both the effects of individual variations and of diet were studied. All three research groups observed that the coefficient of variaton for the excretion of the major and consistently excreted metabolites were large for all of the subjects studied. Extreme dietary alterations produced relatively small changes in the patterns or amounts of metabolites excreted, but large individual within-subject variations were observed [67]. The data of Chalmers et al. [67] therefore indicate that variations in the ranges of excretion depend mainly on individual metabolic alterations rather than on dietary factors. In a survey of 420 normal subjects on an unrestricted diet, quantitative ranges and frequency distribution patterns of the urinary organic acids were assessed [68]. Histograms of excretion values were prepared for all metabolites and could be allocated to four groups: (a) unimodal distributions with detectable values in almost all subjects (examples: tetronic, 2- and 4-deoxytetronic, citric and *cis*-aconitic acids); (b) apparently unimodal distributions with a number of values below the level of detectability (examples: *p*-hydroxyphenylacetic, glucaric and 3-deoxytetronic acids); (c) clearly bimodal distributions, a unimodal sub-group + a block of undetectable values (examples: 3-hydroxyisovaleric, hippuric and glucuronic acids); and (d) irregular distributions with a majority of undetectable values (examples: benzoic, succinic and 2-ketoglutaric acids).

It is important to bear in mind results such as those referred to above when discussing "pathological amounts" of normal metabolites present in healthy and diseased persons.

8. APPLICATION OF GC—MS PROFILING TO DISCOVER NEW METABOLIC DISORDERS

Widespread amino acid screening programmes in the 1950s and early 1960s using paper and ion-exchange chromatography resulted in the discovery of numerous amino acidemias and amino acidurias. In 1963, Klenk and Kahlke [172] used GC and MS methods to separate and identify an unknown metabolite (phytanic acid) present in a patient who had died of Refsum's disease [173]. Since then, gas-phase analytical methods have become increasingly important for diagnosis and studies of metabolic disorders. Chromatographic profiling in general is a complex, time-consuming, highly specialized and expensive technique that is not suitable for mass screening. Apart from research tasks, these methods are therefore usually applied to patients who are particularly suspected of having a metabolic disease, rather than to large population groups. In general, the signs and symptoms to look for include peculiar smells from the body and body fluids of the patients, a hereditary history of similar diseases in the family, lasting metabolic acidoses, mental retardation, failure to thrive, disturbances in pigment development, severe vomiting in early life and involuntary movements [174—176]. Major deviations in the ex-

cretion of end-products (e.g., urea) of the various metabolic pathways, and disagreement between the sums of the amounts of cations (Na^+, K^+, Mg^{2+}, Ca^{2+}, etc.) and anions (Cl^-, SO_4^{2-}, PO_4^{3-}, etc.) in a body fluid (e.g., an "anion gap"), are signals to be aware of.

Chromatographic profiling using GC and GC—MS have resulted, up to early 1977, in the discovery of 23 different, new diseases. In addition, about 50—60 previously described inborn errors can also conveniently be diagnosed by means of GC—MS techniques. In Table 1 the new metabolic disorders are listed, together with the characteristic (disease-specific) compounds that occur in each instance.

In general the diseases shown in Table 1 were recognized because of the occurrence of pathological metabolites, identifiable by means of GC—MS. Subsequent biochemical investigations, such as enzyme studies on biopsies, on cells grown in tissue culture, metabolic studies using stable and/or radioactive isotopes, dietary studies and loading experiments, are required in order to pin-point and, if possible, to treat the metabolic defect. Studies of this character, and the finding of additional cases with the same disorders as listed in Table 1, have been described in numerous publications. In many of these studies, GC and MS were utilized, e.g., to follow changes in the profiles after a loading test. In 1974, Gompertz [177] reviewed inborn errors of organic acid metabolism, and a year later Tanaka [178] prepared an even more comprehensive chapter (with 264 references) on the same topic (disorders of organic acid metabolism). These two excellent publications review in detail what is known about the biochemistry of many of the disorders listed in Table 1, and consider both the first patients and the additional cases that have been reported. Therefore, no attempt is made in this review to go into details about the biochemical defects behind the diseases shown in Table 1.

9. APPLICATION OF GC—MS PROFILING TO INVESTIGATE KNOWN METABOLIC DISORDERS AND OTHER HUMAN DISEASES

Profiling techniques using GC and MS have been used extensively not only to study the new disorders shown in Table 1 (see also refs. 177, 178), but also to diagnose and to obtain more biochemical information on several other previously known metabolic errors and other defined clinical conditions. In books edited by Stanbury et al. [70], Nyhan [205], Hommes and Van den Berg [206] and particularly in refs. 1, 176 and 207 are tabulated numerous inborn errors that can be diagnosed by means of gas-phase analytical methods. Approximately half of the 200 metabolic disorders that are recognized today can be studied by such methods. These techniques are of considerable value for confirming the diagnosis of patients suffering from any one of these diseases. The GC—MS methods are therefore used routinely in several specialized hospital laboratories and other institutions for the examination of specimens from patients suspected of suffering from metabolic disease.

Some of the diseases and clinical conditions that have recently been studied by means of GC—MS techniques are discussed below.

A. Ketoacidosis

This clinical condition has many causes and has been subjected to numerous investigations by means of chromatographic profiling techniques. The volatiles in the urine and breath from patients with diabetic ketosis have been separated and identified, e.g., by Zlatkis et al. [208] and Liebich and Al-Babbili [209] (for a review of volatiles, see ref. 19). Pettersen and co-workers [157, 210—212] and Landaas [213—215] focused their attention on the organic acids. It was found that ketoacidosis leads to increased excretion of adipic and suberic acids [210], whereas the levels of several 3-methyl branched-chain dicarboxylic acids, previously not recognized as normal constituents of human urine [157], were not raised significantly during ketosis. In uncorrected juvenile diabetics there were considerable amounts of adipic and suberic acids, which disappeared on insulin therapy [211]. The formation of the C_6 and C_8 dicarboxylic acids involved an initial omega-oxidation of long-chain fatty acids followed by beta-oxidation [212]. Landaas has shown that ketoacidosis also results in enhanced excretion of 3-hydroxyisovaleric acid [213] and of 3-hydroxyisobutyric and 2-methyl-3-hydroxybutyric acids [214]. These new metabolites stem from the degradation of leucine, valine and isoleucine and accumulate in ketoacidosis because acetoacetate and 3-hydroxybutyrate impair their further metabolic breakdown [215].

B. Lactic acidosis

This condition is known to accompany anaerobic metabolism and is also known to be associated with many diseases (e.g., glucose-6-phosphatase deficiency, fructose-diphosphatase deficiency, methylmalonic acidemia and pyruvate carboxylase and dehydrogenase deficiencies (see review by Tanaka [178] and a recent article [216] describing lactic acidosis in three siblings due to a double enzyme defect). GC—MS has been used to investigate many cases of lactic acidosis and Landaas and Pettersen [217, 218] showed that there is a close relationship between the urinary excretion of 2-hydroxybutyric acid and the occurence of lactic acidosis. The main factor responsible for the increased formation of 2-hydroxybutyric acid appears to be an increased $NADH_2/NAD$ ratio [218] (2-hydroxybutyrate, incidently, is a metabolite also seen in oast-house disease or methionine malabsorption syndrome, and is a normal constituent of amniotic fluid [141]).

C. Maple syrup urine disease (MSUD)

MSUD of various types and degrees of severity is one of the best known and most widely studied metabolic disorders, and has been extensively examined by means of paper chromatography and ion-exchange techniques. Chromatographic profiling using GC—MS methods [79, 97, 98, 219] has yielded additional information on the disease. For instance, clinical attacks result not only in increased excretion of the branched-chain 2-keto and 2-hydroxy acids, but also give rise to an accumulation of all of the metabolites (see above) associated with ketoacidosis and lactic acidosis. Altogether 15

TABLE 1

NEW METABOLIC DISORDERS DISCOVERED BY CHROMATOGRAPHIC PROFILING OF BODY FLUIDS USING GC AND/OR MS METHODS

Disorder	Disease-specific metabolites detected by GC–MS	References*
Refsum's disease** (phytanic acid storage disease)	Phytanic acid	Klenk and Kahlke, 1963 [172]; Eldjarn, 1965 [179]; Steinberg et al., 1965 [180]
Isovaleric acidemia	Isovaleric acid, isovalerylglycine, 3-hydroxyisovaleric acid	Tanaka et al., 1966 [77]
Methylmalonic aciduria		Oberholtzer et al., 1967 [181]; Stokke et al., 1967 [182]; Levy et al., 1970 [183]; Ando et al. 1972 [184]
Type I	Methylmalonic and propionic acids, homocystine and occasionally methylcitric acid	
Type II		
Type III		
Type IV		
Type IV? Methylmalonic and 3-hydroxy-*n*-valeric acidemia	Methylmalonic, propionic, 3-hydroxy-*n*-valeric and odd-chain fatty acids	Stokke et al., 1973 [121]
Propionic acidemia	Propionic, 3-hydroxypropionic, methylcitric, 3-hydroxy-3-methylglutaric and 3-hydroxy-butyric acids and propionylglycine	Hommes et al., 1968 [78]; Gompertz et al., 1970 [185] Barnes et al., 1970 [186]
Type I: biotin-unresponsive		
Type II: biotin-responsive***		
3-Methylcrotonyl-CoA carboxylase deficiency		
Type I: Biotin-unresponsive	3-Methylcrotonylglycine, 3-hydroxyisovaleric acid	Eldjarn et al., 1970 [187]
Type II: Biotin-responsive***	3-Methylcrotonylglycine, tiglylglycine, 3-hydroxyisovaleric, 3-methylcrotonic, methylcitric and 3-hydroxypropionic acids	Gompertz et al., 1971 [188]; Chalmers et al., 1974 [189]
Type III: ?	2-Oxoglutaric and 3-hydroxyisovaleric acids, not 3-methylcrotonylglycine	Finnie et al., 1976 [190]
Pyroglutamic aciduria (5-oxoprolinuria, glutathione	Pyroglutamic acid (2-oxoproline)	Jellum et al., 1970 [81]; Hagenfeldt et al., 1974 [191]

Acyl CoA-dehydrogenase deficiency? (congenital dicarboxylic aciduria)	Saturated C_8–C_{14} dicarboxylic acids, C_{10}–C_{14} *cis*-5-monounsaturated dicarboxylic acids and C_{10} and C_{12} *trans*-3-monounsaturated dicarboxylic acids	Borg et al., 1972 [192]; Lindstedt et al., 1976 [160]
2-Methylacetoacetic and 2-methyl-3-hydroxybutyric aciduria	2-Methylacetoacetic, 2-methyl-3-hydroxybutyric acids and tiglylglycine	Daum et al., 1973 [193]; Hillman et al., 1972 [194]
D-Glyceric acidemia		
Type I	D-Glyceric acid and glycine	Brandt et al., 1974, 1976 [195–197]
Type II	D-Glyceric acid	Wadman et al., 1975 [198]
2-Ketoadipic aciduria	2-Ketoadipic, 2-hydroxyadipic and 2-aminoadipic acids	Wilson et al., 1975 [199]; Przyrembel et al., 1975 [168]
Glutaric aciduria		
Type I	Glutaric, 3-hydroxyglutaric and glutaconic acids	Goodman et al., 1975 [82]; Gregersen et al., 1977 [202]
Type II	Glutaric, (no glutaconic), lactic, isobutyric, isovaleric, propionic, 2-methylbutyric, ethylmalonic and 3-hydroxybutyric acids and several metabolites associated with lactic- and keto-acidoses	Przyrembel et al., 1976 [200]
Carnitine deficiency	Adipic, pimelic and suberic acids	Kapati et al., 1975 [201]
Non-ketotic dicarboxylic aciduria (carnitine deficiency?)	Adipic, suberic and sebacic acids and suberylglycine	Gregersen et al., 1976 [100]
3-Hydroxy-3-methylglutaric aciduria	3-Hydroxy-3-methylglutaric, 3-methylglutaconic, 3-hydroxyisovaleric and 3-methylglutaric acids (not 3-methylcrotonic acid [116, 128])	Faull et al., 1976 [203]; Wysocki et al., 1976 [116]; Faull et al., 1976 [128]
Glyceroluria	Glycerol	McCabe et al., 1977 [204]

*Only the first published references on the first patients are cited.
**Classified and described clinically by Refsum [173] many years before GC and MS methods were used to discover the accumulated metabolite.

***A recent investigation [292] indicates that some of the patients with biotin-responsive propionic acidemia and 3-methylcrotonyl-CoA carboxylase deficiency may suffer from a combined carboxylase deficiency due to a defect in the transport and/or metabolism of biotin rather than the synthesis of the apoenzymes.

metabolites [98] were excreted by MSUD patients during attacks. A glass capillary column was required in order to separate these closely similar compounds. The roles of the newly recognized additional metabolites in MSUD and their possible contribution to the clinical picture are not yet known.

D. Chromatographic profiling of urine from mentally retarded patients

Many of the known inherited metabolic diseases lead to mental retardation and a likely place to start a systematic search for new inborn errors might therefore be in institutions for mentally retarded children. Jellum et al. [111] reported the initiation of such studies in 1971 using a comprehensive GC—MS system capable of detecting organic acids, aldehydes, ketones, amino acids carbohydrates and conjugates. The system was designed primarily for qualitative and semi-quantitative analyses. In 1972, they briefly reported [1] that after analysing urine from more than 450 mentally retarded children no indication of unknown metabolic diseases had been found.

In 1975, Watts et al. [207] reported on a comprehensive study of urinary organic acids in specimens from 1778 mentally retarded patients in comparison with 420 age- and sex-matched controls. Quantitative extraction and determination of the various organic acids were carried out. Approximately 5% of the patients had an abnormal organic aciduria, of which 1% was due to phenylalanine metabolites in cases of phenylketonuria, about 1% was due to increased excretion of benzoic acid and about 1% of the patients showed raised urinary excretion of 2-ketoglutaric and citric acids. Gompertz [220] pointed out that excretion of benzoic acid may be due to bacterial infection of the urine, as Perry and Hansen [103] had shown earlier. If the benzoic acid excreters and the phenylketonuria cases were excluded from the results described by Watts et al. [207], the incidence of organic aciduria in the mentally retarded patients fell to 1.95% [220]. In a reply to Gompertz's letter [220], Chalmers et al. [221]. who were aware of excretion of benzoic acid in urinary tract infections, discussed the occurrence of benzoic aciduria further and the potential effects of drug therapy on the organic acid profiles.

The main conclusion to be drawn from the studies discussed above is that the known organic acidurias do not account for a significant proportion of the children who have survived long enough to be admitted to institutions for mentally retarded patients. On the other hand, it is not unreasonable to assume that many cases of mental retardation patients must be due to an underlying biochemical defect. The problem is the failure of GC—MS to detect these biochemical abnormalities. Gas-phase analytical methods are probable not the most suitable, and the unsatisfactory results obtained by systematic chromatographic profiling emphasize the need for alternative methods, e.g., procedures for detecting changes in the patterns of medium- and/or high-molecular weight body constituents.

E. Stroke patients

Lin and Horning [222, 223] have developed a method for the concurrent analyses of long-chain fatty acids, cholesterol and tocopherols [222] in plasma,

using glass capillary columns. Their method was applied to a study of plasma samples from stroke patients and the results [223] indicated the existence of altered lipid metabolism and altered phosphatidylcholine structures. The changes may be due to depletion of α-tocopherol and linolenic acid.

The clinical value of these profiling techniques is not yet known but, as Lin and Horning suggest [223], it may be possible to define conditions of minimal biochemical risk and high risk of thrombotic disorders by use of these GC analyses.

F. Glycogen storage diseases

Samples from patients with this disease have been examined by GC—MS methods, and elevated levels of C_8—C_{10} dicarboxylic acids were found in the urine [224].

G. Hyperprolinemia

Further studies using GC—MS on patients with hyperprolinemia, type II, showed the presence of considerable amounts of a new metabolite, the glycine conjugate of pyrrole-4-carboxylic acid [225]. The organic acid profile also contained an additional unknown derivative of the pyrrolecarboxylic acid and very large amounts of succinic acid.

H. Formiminoglutamic aciduria

This previously recognized metabolic disorder has been subjected to further studies, and GC—MS proved useful for the identification of the abnormal urinary metabolite (formiminoglutamic acid) [226] and for its determination using multiple ion detection [227]. Analyses showed that this disorder also leads to increased urinary excretion of hydantoin-5-propionic acid [228].

I. Congenital dicarboxylic aciduria

Further studies [160] on samples from a child who had died from a new disorder, possibly Acyl-CoA dehydrogenase deficiency, showed that impaired β-oxidation resulted in the production not only of lactic acid and saturated dicarboxylic acids, but also of a series of unsaturated dicarboxylic acids. Their metabolic origin, methods for their chemical synthesis and GC—MS data have been described [160]. This investigation also showed that normal urine contains small amounts of the same dicarboxylic acids.

J. Prenatal diagnosis of methylmalonic acidemia

The usual procedure for obtaining a prenatal diagnosis involves withdrawal of amniotic fluid, growing of the foetal cells in tissue culture followed by the assay of the suspected enzyme. A direct GC method has been used in the prenatal diagnosis of methylmalonic acidemia by measuring methylmalonic acid in urine and in amniotic fluid from pregnant heterozygous women at risk [74].

K. Jamaican vomiting sickness

This condition has been extensively investigated by GC—MS, particularly by Tanaka [178]. The disease is caused by a plant toxin, hypoglycin A. Urine from intoxicated patients contains methylenecyclopropylacetic, 2-ethylmalonic 2-methylsuccinic and other dicarboxylic acids [229—231].

L. Phenylketonuria

The classical metabolic disorder phenylketonuria (PKU) continues to be studied, now by advanced GC—MS—computer methods. Chalmers and Watts [232] carried out quantitative studies on the urinary excretion of phenylglycollic, 2-hydroxyphenylacetic, phenyllactic, phenylpyruvic, 4-hydroxyphenyllactic and 4-hydroxyphenylpyruvic acids in over 40 cases of PKU. Pollitt [233] found unusual amounts of phenylpropionic acid in the urine of a PKU patient. This acid apparently arose from the action of gut bacteria on poorly absorbed phenylalanine. It was pointed out that differences in gut flora may be a contributing factor to the apparent heterogeneity of PKU. Wadman et al. [234] described a new, probably inherited variant of phenylalanine metabolism in two sisters, 12 and 14 years old, both in normal mental health. They had a permanently increased excretion of the typical PKU metabolites, in spite of normal phenylalanine concentrations in the blood and urine. GC has been used to determine phenylalanine and its metabolites in serum and urine of various hyperphenylalaninemic subjects, their relatives and controls [235]. New chemical-ionization MS methods have also been applied to analyses of samples from PKU patients [236]. GC analyses seem suitable for the detection of heterozygotes for PKU. Profiles are determined after a dose of phenylalanine [237].

M. Gastrointestinal disorders

Van der Heiden and co-workers [238—240] described the excessive urinary excretion of *p*-hydroxyhippuric acid and other *p*-hydroxyphenyl compounds in patients with gastrointestinal disorders. These metabolites originated from intestinal bacterial metabolism of non-absorbed tyrosine, which also appeared to be the precursor of *p*-hydroxyphenylhydracrylic acid [166]. Normal urine contains about 50—100 mg of volatile phenols per day (mainly *p*-cresol, phenol and smaller amounts of 4-etylphenol), all of which are the products of the metabolism of tyrosine in the gut by bacteria. GC methods have recently been used to study the urinary excretion of such phenols in patients with ileostomy, colostomy and diverticular disease [241]. Several patients with gastroenteritis were found to have increased plasma and urine levels of pyroglutamic acid [242]. This acid is the characteristic metabolite in the disease pyroglutamic aciduria (glutathione synthetase deficiency) (see Table 1). The source of this acid in the patients with gastroenteritis, however, proved to be a low-lactose food, Nutramigen, which had been used in treatment of the patients. Certain patients with gastrointestinal dysfunction excrete salicyluric acid even though they do not receive salicylates [91].

N. Disorders related to catecholamine metabolism

Determinations of catecholamines and their metabolites (e.g., homovanillic acid, vanillylmandelic acid and 3-methoxy-4-hydroxyphenylglycol) are of considerable importance in certain psychiatric and neurological disease and in diseases that involve increased activity in the peripheral adrenergic neurons, e.g., hypertension and tumours such as phaechromocytoma and neuroblastoma. GC—MS methods have been extensively used to diagnose and investigate such disorders. The use of stable isotopes in particular has opened up new possibilities for the study of the turnover and metabolism of catecholamines. Sjöquist and coworkers [243, 244] devised mass fragmentographic methods for the determination of 4-hydroxy-3-methoxymandelic acid and 4-hydroxy-3-methoxyphenylglycol in urine, cerebrospinal fluid, serum and brain. Narasimhachari et al. [99] measured homovanillic and isohomovanillic acids, and some additional metabolites from L-DOPA by a similar selected ion monitoring method. Rapid and simple GC procedures for the determination of homovanillic and vanillylmandelic acids [245, 246], 4-hydroxy-3-methoxyphenylglycol [246, 247], 3,4-dihydroxyphenylglycol [248] and 3,4-dihydroxyphenylacetic acid [249] in urine have been described. Wadman et al. [250] reported the application of GC and TLC methods to the study of urinary catecholamine metabolites in normal and reconvalescent children without neurological tumours and in 150 cases with neuroblastoma, ganglioneuroma or phaeochromocytoma. Zambotti et al. [251] used GC—MS to study the catecholamine metabolites in human amniotic fluid, and concluded that the assay of 4-hydroxy-3-methoxyphenylglycol in amniotic fluid is likely to be of importance in the prenatal diagnosis of congenital neuroblastoma. Free and conjugated 3-methoxy-4-hydroxyphenylglycol and vanillylmandelic acid in human ventricular fluid have provided some quantitative information on the role of the latter metabolite in human central nervous system catecholamine metabolism. Selected ion monitoring was used in this study [252].

O. Respiratory distress syndrome

Assessment of human foetal lung maturity in utero is based on the determination of the lung surfactant in the amniotic fluid. This may be expressed as lecithin or as the ratio of lecithin to sphingomyelin (L/S ratio). The most widely used methods involve extraction of the phospholipid fraction from amniotic fluid, followed by TLC [253]. L/S ratios above 2.0 indicate adequate maturity and little chance that the newborn child will develop respiratory distress syndrome. Several reports, however, indicate that L/S ratios may be of doubtful prognostic value in diabetic pregnancies, and in the critical region with L/S ratios around 1.5—2.0.

GC methods have therefore been taken into use to measure more accurately the fatty acids derived from lecithin hydrolysis. Rapid and convenient transesterification of aminotic lipid extracts, using sodium methoxide and/or methanol—boron trifluoride prior to GC have been used by several authors, e.g. by Lindback [254-256] and by Cooper and Brush [257] who also used MS.

Since dipalmitoyl lecithin is the major alveolar surfactant, GC methods for the specific measurement of lecithinpalmitic acid in amniotic fluid have been worked out. In the latest one of these [258] a hexane—2-propanol—sulphuric acid system was used to obviate major interferences from triglycerides and free fatty acids. Lecithin palmitic acid values, as determined by GC, exceeding 8 mg per liter of amniotic fluid, indicate that the infants are unlikely to develop respiratory distress syndrome. Results obtained by the above groups and other investigators [259, 260] indicate that amniotic fluid lipid profiles may be of some clinical value in asessing maturation of the foetal lung.

P. Bacterial and viral infections

The first attempts to classify microorganisms by GC profiling techniques were made by Abel et al. in 1963 [261]. Since then, many investigators have established that GC and GC—MS, particularly if combined with computer statistics and numerical taxonomy, are valuable supplementary methods in bacterial and viral classification. Two principally different approaches were made. The first involves the analysis of the chromatographic profiles of the microorganisms and their growth environment after in vitro cultivation and isolation. This approach has proved successful and is in common use. Comprehensive work-up procedures for the determination of, e.g., fatty acid profiles and carbohydrate profiles have been described (e.g., refs. 22, 262 and 263). Pyrolysis methods are also much used (e.g., ref. 47).

The second approach involves the direct analysis of the infected material, i.e., body fluids or tissues, without cultivation of the infectious agents. This approach was introduced by Mitruka et al. [264] and is based on the detection of bacterial and viral metabolites among a multitude of host specific compounds. The technique appears to be very attractive owing to its rapidity, and is under consideration by several laboratories [146, 265—267]. Problems regarding the unstandardizable host background have not yet been solved. Mitruka [22] recently edited a book on the application of GC in microbiology in which all work prior to 1975 is discussed. The latest overview of the topic was prepared by Jantzen [263].

Q. Miscellaneous

Although it is outside the scope of this paper to review the GC—MS analysis of steroids, carbohydrates, amino acids and peptides, it seems appropriate at this stage to emphasize that many metabolites belonging to these chemical classes are disease-related and therefore of diagnostic value. Increased urinary excretion of oligosaccharides and/or glycopeptides is found in the diseases aspartyl-glucosaminuria, mannosidosis, glycogen storage disease types II and III and GM_2-gangliosidosis. GC—MS profiling techniques have been developed to diagnose and study these disorders (e.g., refs. 268, 269) and some other lysosomal diseases have also recently been studied by GC—MS (e.g., ref. 270). Carbohydrate profiles have been recorded in seminal fluid from normal and sterile men [29].

Profiling of amino acids and peptides for diagnostic purpose is carried out

mainly by methods other than GC—MS, e.g., thin-layer, paper and ion-exchange chromatography. Some recent advances in GC—MS methodology, particularly on-column derivatization [107], the use of direct chemical-ionization MS [236] and the use of deuterated amino acids as internal standards in conjunction with computerized GC—MS systems [271—273], have led to rapid, highly sensitive and specific alternatives to the traditional methodology. The new methods have been used to study patients with phenylketonuria, cystinuria and maple syrup urine disease [236], and to study a child [272] and a man [274] with defects in collagen metabolism. Both patients excreted considerable amounts of dipeptides, particularly glycylproline. Such dipeptides may be associated with several disorders [275], e.g., prolidase deficiency [272].

The profile approach to the study of inborn errors of metabolism is also relevant to steroid analysis. Several defects in steroid metabolism, e.g., 3β-hydroxy-steroid dehydrogenase deficiency [276], steroid 21-hydroxylase deficiency [277] and congenital adrenal hyperplasia [278], have been studied by GC—MS methods. The major advance in recent years has been the introduction of open-tubular glass capillary columns, which permit the separation of all major physiologically occurring steroids. Steroid profiles, using high-resolution separation methods, have been obtained from normal males and pre- and post-menopausal females and from patients with congenital adrenal insufficiency, adrenal tumours and Cushing's disease [279, 280]. Advanced computerized GC—MS equipment [279, 281] and semi-automated GC methods [280] greatly facilitate such studies.

10. USE OF STABLE ISOTOPES

Many countries are now reluctant to permit the administration of radioactive isotopes to human patients. The use of stable isotopes and GC—MS for in vivo studies on metabolic pathways in healthy and diseased states is an alternative approach that is likely to increase rapidly in importance. Several international conferences on stable isotopes have already been arranged. A considerable number of deuterium-labelled and ^{13}C-labelled metabolites are already commercially available, and new compounds are regularly added to the various catalogues. A review of the biomedical applications of stable isotopes has recently been published by McCloskey [282]. Curtius et al. [283] administered deuterium-labelled amino acids to patients with phenylketonuria, hyperphenylalaninemia and oligophrenia of unknown genesis. More recently they have made an elegant study of the metabolism of tyrosine in the human intestine by means of stable isotopes and GC—MS [284]. Mamer and Tjoa [285] used a deuterium-labelled precurser to explain the production of a new acidic metabolite in normal human urine, viz., 2-ethylhydracrylic acid [163]. Apparently it is produced in a new, minor catabolic pathway from isoleucine. Stable isotopes and GC—MS techniques have been valuable, e.g., for studies on steroid metabolism (e.g., ref. 286), for investigations on glucose metabolism (e.g., ref. 287), in numerous experiments on drug metabolism (e.g., ref. 288), for studies of nitrogen retention in growth hormone deficient children [289] and for studies on the in vivo synthesis of the "essential" amino acid valine from 2-keto[^{13}C]isovalerate [290]. Stable isotope-labelled compounds

are particularly suitable for pharmacokinetic studies, as recently shown by Horning and co-workers [288, 291].

Selected ion monitoring (SIM) is today a widely used method for the quantitative and specific determination of drugs and their metabolites, steroids and biogenic amines and their metabolites. The technique has been introduced as a definite or absolute reference method in clinical chemistry, and cholesterol, triglycerides, urea, glucose, cortisol, progesterone and testosterone can now be determined in plasma or serum with high accuracy [293]. All of these methods utilize molecules labelled with ^{2}H, ^{13}C, ^{14}C or ^{15}N as internal standards. Apart from the determination of catecholamine metabolites (see p. 451), several of which are organic acids, very few other organic acids have so far been quantitated by SIM and stable isotope-labelled internal standards. It is to be expected that this measurement will be achieved in the future.

11. CONCLUSIONS

It is evident from the many results reviewed here that the profiling approach to the study of body fluids has already become a valuable method in biomedicine. It is to be expected that progress will continue rapidly, and that high-resolution separation methods (e.g., with capillary columns) will increase the potential of the approach even further. The development of advanced computer methodology opens up new possibilities, not only for the study of gross alterations, but also for the detection of more subtle changes in the profiles. Information of this type may throw new light on metabolic processes in normal and diseased states. The use of stable isotopes and profiling methods is also likely to expand in the future.

However, numerous problems remain to be solved and much information is still lacking. For example, most of the work has so far been concentrated on urine profiling, and much less on other body fluids such as serum and spinal fluid. Hardly any work has been done on tissue profiling, although it seems reasonable to assume that much information could be gained by applying advanced GC—MS to the analysis of such specimens. One can only hope that the methodology will be advanced so as to be able to handle 1—3 mg of tissue biopsies.

Apart from technical difficulties, artifacts and pitfalls, one is also concerned with the problem of identifying all of the normal peaks that occur in the various chromatograms. For example, when the organic acids in human urine are separated on glass capillary columns, less than one third of the peaks can be identified. Slowly but surely, more of these unknown peaks are likely to be identified, sometimes perhaps with surprising results.

12. SUMMARY

This review summarizes recent advances in the application of gas chromatography and mass spectrometry to the study of human diseases. Emphasis is placed upon the organic acid profiles of the various body fluids. Methods for sample work-up prior to separation and mass spectrometric analysis are reviewed, and artifacts and pitfalls are discussed. Organic acid profiles, obtained

with packed or capillary columns attached to mass spectrometers with or without computer systems, have led to the discovery of new normal metabolites, new metabolic disorders, and to new knowledge about a number of other diseases. Stable isotopes and gas chromatography—mass spectrometry are suitable for quantitative analysis of many compounds in the body fluids, and well suited for investigation of metabolic pathways.

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