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Application of mass spectrometry and metabolite profiling to the study of human diseases

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Many human diseases result in characteristic changes in the biochemical composition of the cells and the body fluids. The profiling approach, with gas chromatography–mass spectrometry (g.c.–m.s.) and computer handling of the data, are suitable for detecting such changes, e.g. the production of abnormal metabolites in a patient. The methods can be used to diagnose and study about 100 different metabolic disorders, and have resulted in the discovery of 25 new inborn errors of metabolism. Other diseases, such as ketoacidosis, lactic acidosis, bacterial and viral infections, rheumatoid arthritis and cancer, are currently studied by means of g.c.–m.s. methods.
  Many difficulties and pitfalls are attached to the investigation of human diseases by g.c.–m.s. Dietary variation, drug intake and artefacts produced during chemical pretreatment of the samples, and lack of knowledge of the normal constituents seen in the various profiles, are factors to consider. Despite many problems, the methods have proved to be valuable analytical tools in the clinical laboratory. It is likely that the new developments in mass spectrometry technology (e.g. automatic computer evaluation of the g.c. profiles, automated pyrolysis–m.s., high resolution g.c.–m.s.) will make the methods play increasingly important roles in the biomedical field.

Introduction

Many human diseases result in characteristic changes in the biochemical composition of the cells and the body fluids. Combined gas chromatography – mass spectrometry (g.c.–m.s.) with data systems are suited to detect such changes, e.g. the accumulation of organic acids in blood and urine. The finding of abnormal metabolites and/or elevated or reduced levels of normal compounds in patients, often gives clues to the nature of the disease. This 'metabolic profiling' approach is now frequently used to diagnose and study metabolic disorders as well as to obtain further information on several other human diseases (for a recent review see Jellum 1977). In the present report some of the recent results obtained in this field of mass spectrometric application will be reviewed.

Instrumentation

  G.c. alone may give useful information on the accumulation or excretion of metabolites, but a mass spectrometer is required to obtain positive identification. The analytical potential of combined g.c.–m.s. is obviously further enhanced by computer attachment. The g.c. columns themselves have also been improved and perhaps the most important change seen over the last years is the availability and use of wall-coated thermostable glass capillary columns. Such columns give not only much better resolution and therefore more information, but also improved sensitivity (sharper peaks, less adsorption).

  The computer part of the g.c.–m.s. equipment may give valuable aids in a variety of ways. It may be used to operate the mass spectrometer as well as for data acquisition. The technique

of 'mass chromatography', where the data are searched for a given set of fragments, is very useful to control whether a certain metabolite is present in a given specimen. The technique can be used to discover new metabolites with certain structural features in common, e.g. we recently used mass chromatography to demonstrate the presence of several pyrrole–carboxylic acid conjugates in specimens from a patient with hyperprolinaemia type II (Applegarth *et al.* 1977). The computer may aid the identification of unknown metabolites, again in many different ways (for a review see Burlingame *et al.* 1978). The simplest way is to perform a computer matching of unknown mass spectra against files of reference mass spectra, placed in local or distant computers which can be reached through international computer networks. A total of about 80 000 mass spectra (many duplicates) are available through several commercial collections, of which the one entitled *Mass spectra of compounds of biological interest* (Markey *et al.* 1975) is perhaps of particular interest in the field of biomedical applications. Software operated selected ion monitoring and the use of stable isotope-labelled internal standards are now becoming increasingly important in the biomedical field for quantitative analyses.

It is often desirable to be able to detect not only gross changes but also more subtle differences in the various chromatographic profiles. Pattern recognition methods and several systems based on retention indices and mass spectral data (Nau & Biemann 1974; Sweeley *et al.* 1974) are in operation for this purpose. It is also possible to use a computer for the automatic recognition of unusual metabolites and to detect the lack of normal constituents (Jellum *et al.* 1975). In the latest publication by Sweeley and coworkers (Gates *et al.* 1978) more than 100 components in a typical mixture of urinary organic acids are automatically identified and quantitated at a rate of one compound each 6 s.

Sample types, sample work-up, patient selection

Urine is by far the most widely used physiological fluid for g.c.–m.s. profiling analyses. When quantitative data are not required, morning samples are preferred, otherwise 24 h urine is used, or the results are expressed relative to the creatinine content (but with caution). Serum is also frequently used, e.g. to confirm the presence of increased amounts of acid in cases of the acidemias. Amniotic fluid and cerebrospinal fluid have also been subjected to many analyses by g.c.–m.s. Prenatal diagnosis of methylmalonic acidaemia for instance, can be made by the direct measurement of methylmalonic acid in amniotic fluid from heterozygous women at risk (Nakamura *et al.* 1976). Respiratory distress syndrome can be predicted by g.c.–m.s. analyses of dipalmitoyllecithin in amniotic fluid (t.l.c., however, is more frequently used for this purpose). Disorders of catecholamine metabolism can conveniently be studied by selected ion monitoring methods (mass fragmentography) applied to cerebrospinal fluid. Other body fluids, e.g. seminal fluid, synovial fluid, saliva, sweat, tears, gastric juice and dialysis fluid have only to a limited extent been examined by g.c.–m.s. (Jellum 1977). The possibility of analysing small tissue biopsies (obtained by needle techniques) by capillary g.c.–m.s. (Goodman *et al.* 1977) opens an interesting area, particularly within the field of inborn errors of metabolism. In this way one can approach the affected tissues directly, and learn about the metabolic situation inside the cells.

The chemical classes of metabolites which can be analysed by g.c.–m.s. and which are of interest in studies on human diseases include alcohols, aldehydes, ketones, carbohydrates, amino acids and peptides, nucleic acid derivatives, biogenic amines, prostaglandins, steroids,

bile acids, organic acids and fatty acids. It is particularly in the field of organic acidaemias that the g.c.–m.s. methods have proved to be of great diagnostic value. Amino acidaemias and disorder of sugar metabolism, for instance, are still more conveniently screened for and diagnosed by paper chromatography, t.l.c. and ion exchange methods, and the g.c.–m.s. methods are usually only taken into use when further characterization of these disorders is required. As it is outside the scope of this article to discuss the sample work-up with respect to all the above different types of compounds, the reader may instead consult recent reviews (Jellum 1977; Burlingame *et al.* 1978; Cram & Risby 1978). It should be emphasized, however, that no matter what sample work-up and derivatization method one selects, it is very important to learn the limitations and pitfalls associated with the particular procedures that have been chosen.

As chromatographic profiling by g.c.–m.s. is a complex, time-consuming, highly specialized technique, it is not suitable for mass screening. In work which is not typical research, e.g. the routine application of the technique to diagnoses of metabolic disorders, some selection of the patients is required. By now it is clear that the g.c.–m.s. methods should be applied to patients who have one or more of the following signs and symptoms: peculiar smells from the body and body fluids, a hereditary history of similar diseases in the family, lasting metabolic acidosis, mental retardation, failure to thrive, disturbances in pigment development, severe vomiting in early life and involuntary movements. Major deviations in the excretion of end-products (e.g. urea) and disagreement between the sums of the amounts of cations and anions in a body fluid (e.g. an 'anion gap') are also signals to be aware of.

APPLICATION TO DIAGNOSES AND STUDIES OF HUMAN DISEASES

With selection of patients along the above lines and by making use of the g.c.–m.s. techniques discussed above, about 25 new metabolic diseases, mainly organic acidaemias, have been discovered on a world-wide basis. In addition, about 60–70 previously described enzyme defects can also conveniently be diagnosed by means of g.c.–m.s. (for references see Jellum 1977). In general the new diseases were recognized because of the occurrence of pathological metabolites identifiable by means of g.c.–m.s. Subsequent biochemical investigations, such as enzyme studies on biopsies or on cells grown in tissue culture, metabolic studies with the use of stable and/or radioactive isotopes, dietary studies and loading experiments, are required in order to pinpoint and, if possible, to treat the metabolic defects. Also, in studies of this character, g.c.–m.s. is a valuable analytical tool (for reviews see Gompertz (1974) and Tanaka (1975)).

The g.c.–m.s. methods have been used to reinvestigate a number of previously described metabolic diseases, e.g. ketoacidosis, lactic acidosis, maple syrup urine disease, glycogen storage disease, phenylketonuria and tyrosinaemia. In several instances important new knowledge about the diseases have been obtained. Hereditary tyrosinaemia, for example, has for many years been believed to be caused by a defect in the further metabolism of *p*-hydroxy-phenylpyruvate which is formed from the amino acid tyrosine. In an elegant study Lindblad *et al.* (1977) used g.c.–m.s. to show that tyrosinaemia patients excrete, in addition to the well-known tyrosine metabolites, considerable amounts of a new metabolite, succinylacetone. Subsequent enzyme studies revealed a defect at an entirely different step from that previously recognized, namely an impaired degradation of fumaroylacetoacetate, which is the precursor of succinylacetone (Lindblad *et al.* 1977). This information in turn has been used to improve

the dietary régime used for treatment of the disease (extra supply of cysteine which may become depleted from the body of the patients owing to interaction with fumaroylacetoacetate (Slørdahl *et al.* 1978)).

Although g.c.–m.s. at present is most widely used to study the organic acidaemias, it should be pointed out that several laboratories are using the methods for analyses of volatiles, steroids, carbohydrates, amino acids and peptides and are thus able to diagnose metabolic disorders related to these compounds. Increased urinary excretion of oligosaccharides and/or glyco-peptides is found in the diseases aspartylglucosaminuria, mannosidosis, glycogen storage disease types II and III and GM_2-gangliosidosis. G.c.–m.s. profiling techniques have been developed to diagnose and study these disorders and some other lysosomal diseases (for references see Jellum 1977). Some recent advances in g.c.–m.s. methodology, particularly on-column deri-vatization, the use of direct chemical-ionization m.s. and the use of deuterated amino acids as internal standards in conjunction with computerized g.c.–m.s. systems, have led to rapid, highly sensitive and specific alternatives to the traditional methodology for analyses of amino acids and peptides. The new methods have been used to study patients with phenylketonuria, cystinuria and maple syrup urine disease, and to study patients with defects in collagen meta-bolism (see, for example, Faull *et al.* 1976).

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-hydroxysteroid dehydrogenase de-ficiency, steroid 21-hydroxylase deficiency and congenital adrenal hyperplasia, have been studied by g.c.–m.s. Steroid profiles from high-resolution capillary columns 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 (for references see Jellum 1977).

The use of stable isotopes and g.c.–m.s. for in-vivo studies on metabolic pathways in inborn errors of metabolism is an approach which rapidly is becoming more important, as many countries are now reluctant to permit the administration of radioactive isotopes to human patients. A considerable number of deuterium-labelled and ^{13}C-labelled metabolites are com-mercially available and many interesting studies on metabolic errors have been reported (e.g. Curtius *et al.* 1976). Deuterium-labelled amino acids, for instance, have been administered to patients with phenylketonuria, tyrosinaemia and hyperphenylalaninaemia. The metabolism of the labelled compounds has been followed by g.c.–m.s. It is likely that studies of this type will yield new information on many diseases.

The metabolic disorders discussed above are in general rare diseases, and one specialized g.c.–m.s. laboratory can in principle serve a large community. A different situation would arise if the profiling approach could be used, e.g. for early diagnosis of cancer. Some research in this area is currently taking place. For instance, the Norwegian Cancer Society has since 1973 collected and stored at $-20\ °C$ serum samples from about 20 000 persons who are donating blood at regular intervals. In the 5 years that now have passed, almost 400 persons have developed some kind of cancer. Since deep-frozen serum now is available from the same person, e.g. 3, 2 and 1 year before disease was diagnosed, as well as after diagnosis was made but before any treatment was started, the material offers a unique opportunity to detect early signs of disease. Capillary g.c.–m.s., pyrolysis m.s. and field-desorption m.s. as well as other methods (e.g. two dimensional electrophoresis) are now being used in an international cooperative study aimed at detecting early signs of cancer. No results are as yet available.

Mass spectrometric techniques have also been employed in studies of male infertility, and

over 20 carbohydrates have been identified in human seminal fluid (Størseth *et al.* 1978). No correlation between these compounds and infertility was found. Capillary g.c.–m.s. methods suitable for determining the organic acid profile of synovial fluid have recently been developed (Heininger *et al.* 1978). Fluid from normal joints and from patients with rheumatoid arthritis showed a similar qualitative pattern of acids (mainly fatty acids) resembling the pattern found in serum. The concentrations of organic acids in the abnormal synovial fluids were, however, 5–10 times higher than those in synovial fluid from the normal joints. This probably contributes to a reduced lubricating efficiency of the synovial fluid in patients with rheumatoid arthritis.

Finally, it should be mentioned that diseases due to bacterial and viral infections also are being studied by different mass spectrometric techniques. Thus, the first attempts to classify microorganisms by g.c. profiling techniques were made by Abel *et al.* (1963). Since then, many investigators have established that g.c. and g.c.–m.s., particularly if combined with computer statistics and numerical taxonomy, are valuable supplementary methods in bacterial and viral classification. Two principally different approaches are taken. 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 frequent use. Comprehensive work-up procedures for the determination of, for example, fatty acid profiles and carbohydrate profiles have been described (see, for example, Mitruka 1975). The recent progress in pyrolysis-m.s. with laser or Curie-point flash heating combined with multivariate data analysis appears to be very promising in modern microbiology (Meuzelaar *et al.* 1974). 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 (1975) and is based on the detection of bacterial and viral metabolites among a multitude of host specific compounds. The technique appears to be attractive, but problems regarding the unstandardizable host background have not yet been solved.

Current difficulties and problems

Although g.c.–m.s. is at present used successfully by several laboratories and hospitals for diagnosis and studies of human diseases, e.g. inborn errors of metabolism, it is important to point out that the analytical approach is unfortunately associated with numerous problems and difficulties. First, there are problems with collection and storage of the samples. Depending on the container used, contamination may occur from plasticizers from rubber stoppers and from added preservatives and anticoagulants (e.g. heparin contains benzyl alcohol as a stabilizer). Secondly, numerous problems exist with sample work-up and with the derivatization methods, e.g. formation of artefacts and multiple derivatives. The production of artefacts (e.g. crotonic acid from β-hydroxybutyric acid) and the formation of unexpected compounds in the g.c. column (e.g. 5-hydroxycoumaran from homogentisic acid and 3-methylcrotonic acid from 3-hydroxyisovaleric acid) may lead to serious errors. Decarboxylation reactions (e.g. of methylmalonic acid to propionic acid and of phenylpyruvic acid to phenylacetic acid) during sample work-up and g.c. may lead to erroneous results. New compounds formed by transesterification processes and artefacts (e.g. benzoic acid) produced by bacteria are potential sources of error. Perhaps the most serious problems arise from artefacts due to dietary factors and intake of drugs. Particular attention should be paid to drugs that are metabolized to give

organic acids that one normally associates with metabolic disorders. Thus, Gompertz *et al.* (1977) studied the anticonvulsant Valproate, which leads to increased urinary excretion of propionic acid, 2-oxo-dipropylacetic acid and 2-(*n*-propyl)glutaric acid. In general, many problems can be avoided if correct and complete information about drug intake always accompanies the samples submitted for chromatographic profiling.

Other problems concern the normal metabolic profiles, or rather the lack of knowledge about them. The quantitative ranges and effects of individual variation and diet on the profiles is one aspect (see, for example, Chalmers *et al.* 1976), and the actual identification of many g.c. peaks is another aspect. All major and also many minor peaks seen in the chromatograms from urine, serum and to a certain extent from cerebrospinal fluid and amniotic fluid, have been identified. However, as one switches from packed to capillary columns, it becomes evident that every peak from a packed g.c. column is composite and consists of at least three or more components. The number of unknown peaks in capillary chromatograms of normal human body fluids is therefore considerable, and much work remains to identify them all. If tissue biopsies and other more rarely used body fluids also are considered, the lack of knowledge about the normal patterns is striking. It is clear that new, normal metabolites are and will be reported regularly and it would be advantageous to coordinate this type of information on an international basis.

Concluding remarks

The application of g.c. and m.s. methods to investigations of human diseases, particularly the inborn errors of metabolism, have during the last decade proved to be of considerable value. In several hospitals the methods are now used on a routine basis for studies on inborn errors and for pharmacological and toxicological studies. The trend shows that more g.c.–m.s. instruments are now going into hospitals. Most of these places are using packed g.c. columns, but it is likely that this will change in the near future, as modern glass capillary columns offer many advantages. It is also probable that some kind of computer evaluation of the complex chromatographic profiles (e.g. automatic identification and quantification of compounds (see, for example, Gates *et al.* 1978)) will become increasingly important.

A time-consuming step in most of today's procedures is the sample pre-treatment. Although several methods, including extraction, ion exchange and liquid chromatography, are used for isolation of metabolites, and different ways of making volatile derivatives are employed, it should be possible to automate these steps. Automatic sample pretreatment combined with automatic injection into the g.c.–m.s. instrument would mean a great improvement.

G.c.–m.s. profiling techniques are slow methods, unsuitable for mass screening. Pyrolysis–m.s.–computer methods (Meuzelaar *et al.* 1974) and field ionization–m.s.–computer methods (Anbar *et al.* 1976), on the other hand, are rapid procedures which have the capacity to analyse many samples. Perhaps a good solution would be to use these methods for screening purposes, and whenever obvious and significant deviations from the multifragmentation pattern are discovered one could turn to capillary g.c.–m.s. methods for identification of the pathological metabolites.

In addition, the combination of liquid chromatography and m.s. is an approach which is likely to become useful in the study of human diseases, particularly in the analysis of labile metabolites and compounds of intermediate molecular mass. Multicomponent analyses of proteins and peptides have until now been impossible, but also this situation is rapidly changing.

Two-dimensional electrophoresis (O'Farrel 1975; Anderson & Anderson 1977) with computer evaluation of the data is now capable of separating several hundred, perhaps over 1000 proteins in serum and tissue samples (Anderson & Anderson 1978). Methods of the above types, combined with capillary g.c.–high resolution m.s.–computer (Burlingame *et al.* 1976) have extremely high analytical potentials, which when applied to the study of human diseases are likely to yield new and valuable information.

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