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APPLICATION OF GLASS CAPILLARY-COLUMN GAS CHROMATOGRAPHY–MASS SPECTROMETRY TO THE STUDIES OF HUMAN DISEASES

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SUMMARY

Open-tubular glass capillary columns have been used in gas chromatography in combination with mass spectrometry (GC–MS) and computer methods to study human diseases. Patients with maple syrup urine disease excrete not only α-keto and α-hydroxy acids but also six other metabolites which hitherto have been overlooked. The GC–MS methods demonstrated that a group of patients suffering from hereditary progressive loss of hearing have an impaired metabolism of leucine, leading to the accumulation of 3-hydroxyisovaleric acid and 3-methylcrotonylglycine. GC using the capillary columns proved suitable for mapping of the carbohydrate profile of human seminal fluid and for the analyses of organic compounds accumulating in human adipose tissue. The high resolving power and long life of the glass capillary columns suggest that they will be valuable in the diagnosis and studies of human disorders.

INTRODUCTION

The value of gas chromatography (GC) and mass spectrometry (MS) in the diagnosis of and studies on human diseases is now well recognized. Blood and urine samples from "problem cases", for instance, are routinely examined by GC–MS methods in several hospital laboratories throughout the world (*e.g.*, refs. 1–3). The analytical procedures nearly always involve the use of packed GC columns, although it has been realized for a long time that many components of the highly complex biological fluids are not resolved on these columns.

The high resolving power of capillary columns have been known for nearly 20 years[4], but only during the last few years has it been possible to prepare thermostable glass open-tubular capillary columns that have proved suitable for separation of biological specimens (*e.g.*, refs. 5–10). Columns of this type have recently become commercially available and have now been incorporated in our GC–MS–computer system[11,12], which is used for investigations of human diseases.

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We report here some of the results obtained using capillary column GC–MS for the multicomponent analysis of body fluids and tissues in normal and diseased states.

EXPERIMENTAL

Apparatus

The combined GC–MS–computer instrument consisted of a Varian 1400 gas chromatograph, a Varian 112 double-focusing mass spectrometer and a Spectrosystem 100 MS computing system (Varian-MAT, Bremen, G.F.R.). The gas chromatograph was fitted with a variable split injection system and a 25-m glass capillary column (LKB, Stockholm, Sweden), coated with either SE-30 or SP-1000. The number of theoretical plates on these columns was in the range 80,000–90,000. The mass spectrometer was fitted with dual turbomolecular pumps, allowing direct inlet of the effluent from the capillary column into the ion source, which was operated at 70 eV. The computing system included automatic computer evaluation of the chromatographic profiles (CASAC, first version[13]) and search programs for matching of unknown mass spectra against a library containing 77,120 entries[12].

Sample work-up and gas chromatography

Details of our routine analytical procedures, which involve several extractions (with diethyl ether) and derivatization methods (with diazomethane and trifluoroacetic anhydride), have been published earlier[11]. Additional methods were developed for the analyses of keto acids, in which use was made of oxime-TMS derivatives[14]. Analyses of carbohydrates in seminal fluid involved the use of a filtration technique for deproteinization and ion exchange for removal of charged compounds before derivatization with methoxylamine and with BSTFA silylating reagent (see legend to Fig. 4). Analyses of human adipose tissue included saponification with ethanolic sodium hydroxide and with aqueous sodium hydroxide and subsequent removal of the neutral, unsaponified material from the soap by extraction with diethyl ether.

The gas chromatographic conditions were, unless otherwise stated, the following: splitting ratio, 1:60; temperature of injection port, 245°; column temperature programmed from 50° to 270° at a rate of 6°/min; flow-rate of carrier gas (helium), 1.0 ml/min. Uptakes of mass spectra were normally effected in the repetitive scan mode, with a total scan time of about 2 sec for every spectrum (mass range 20–500) including the fly-back time.

RESULTS AND DISCUSSION

Organic acids in the urine

A number of metabolic disorders are characterized by increased excretion in the urine of one or more organic acids. The systematic search for carboxylic compounds in the urine from patients has consequently become of considerable importance, and packed-column gas chromatography (and mass spectrometry) has so far led to the discovery of more than 15 new diseases. Fig. 1 shows the separation of urinary organic acids from a patient with ketoacidosis on a packed column and on a capillary column. Comparison of the two chromatograms demonstrates that every

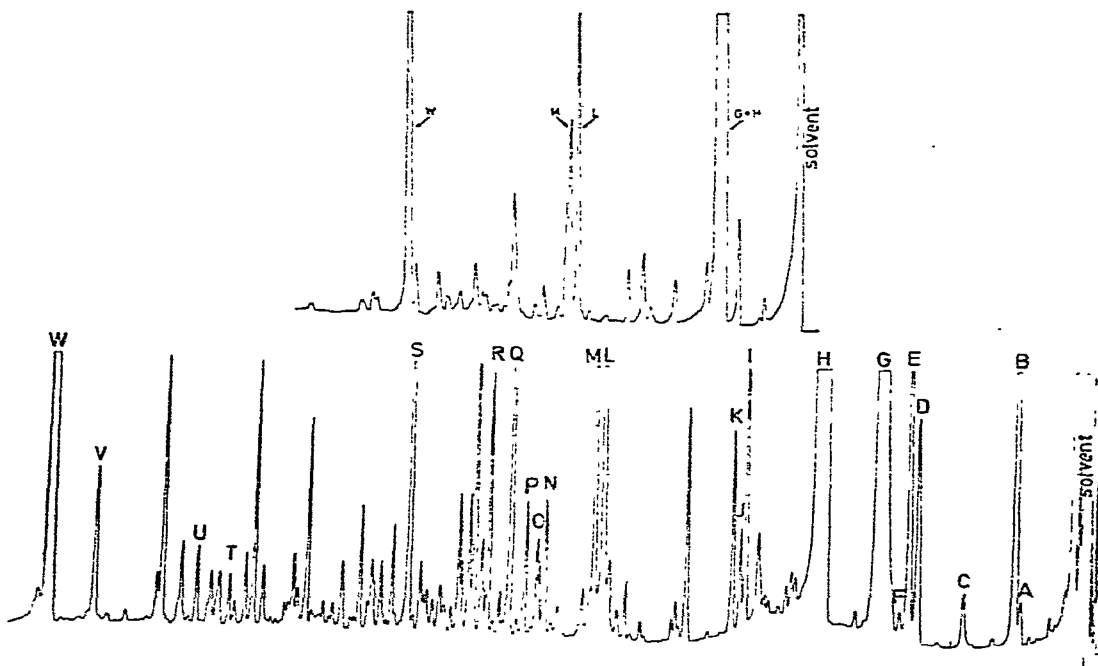

Fig. 1. Organic acids in the urine of a ketoacidotic patient. Top chromatogram: separation on a packed GC column (6 ft. × 1/4 in., 10% OV-17 on Gas-Chrom Q, temperature programmed from 80° to 300° at a rate of 8°/min). Bottom chromatogram: separation on a 25-m SP-1000 glass capillary column (GC conditions as described in text). The organic acids were extracted with diethyl ether and converted into methyl esters with diazomethane prior to GC. The top chromatogram was recorded with an FID and the bottom chromatogram with the mass spectrometer as detector, explaining the difference in response. Peaks: A = 2-hydroxyisobutyric acid; B = artifact from 3-hydroxybutyric acid; C = lactic acid; D = 3-hydroxyisovaleric acid; E = 2-hydroxybutyric acid; F = 2-hydroxyisovaleric acid; G = acetoacetic acid; H = 3-hydroxybutyric acid; I = 3-hydroxyisobutyric acid; J = succinic acid; K = benzoic acid; L = adipic acid; M = 3-methyladipic acid; N = pimelic acid; O = 3-methylpimelic acid; P = cyclopropaneadipic acid; Q = eicosane (internal standard); R = suberic acid; S = thymol (added preservative); T = diphenylamine (from the diethyl ether); U = homovanilic acid; V = *p*-hydroxyphenylacetic acid; W = hippuric acid.

peak from the packed column must consist of a mixture of several components. It is obvious that valuable information may be hidden underneath these composite peaks, and that metabolites of importance for a particular disease may easily be overlooked.

With this in mind, we undertook a re-investigation of certain metabolic disorders, using capillary-column GC–MS. The first disease to be considered was maple syrup urine disease (MSUD), which arises as a result of a defect in the degradation of the branched-chain amino acids leucine, isoleucine and valine. It has previously been shown by a variety of methods, including packed-column GC, that urine from these patients contains three branched-chain α-keto acids, their corresponding hydroxy acids and usually also lactic acid and β-hydroxybutyric acid. GC using capillary columns (Fig. 2) demonstrates that the urine from these patients contains in addition six other pathological metabolites which hitherto have been overlooked. As the cause of the clinical symptoms in MSUD still remains unclear, it may be that some

Fig. 2. Metabolites in the urine of an untreated patient with maple syrup urine disease. An acidified aliquot of the urine was extracted with ethyl acetate and the extract was derivatized with hydroxylamine in pyridine followed by BSTFA treatment. Gas chromatographic conditions: injection temperature, 250°; splitting ratio, 1:12; capillary column (25-m SE-30) programmed from 80° to 110° at a rate 0.5°/min. The hatched peaks belong to solvent and reagents. Peaks: 1 = lactic acid; 2 = 2-hydroxyisobutyric acid; 3 = 2-hydroxybutyric acid; 4 = pyruvic acid; 5 = 3-hydroxybutyric acid; 6 = 2-hydroxyisovaleric acid; 7 = 2-ketobutyric acid; 8 = 2-methyl-3-hydroxyisobutyric acid; 9 = 3-hydroxyisovaleric acid; 10a and 10b = 2-ketoisovaleric acid; 11 = acetoacetic acid; 12 = 2-hydroxyisocaproic acid; 13 = 2-hydroxy-3-methylvaleric acid; 14a = L-2-keto-3-methylvaleric acid; 14b = D-2-keto-3-methylvaleric acid; 15 = 2-ketoisocaproic acid. Peaks 2, 3, 7, 8, 9 and 14b have not previously been associated with MSUD. Internal standard = malonic acid (i.st.).

of the metabolites revealed in Fig. 2 may contribute to the symptoms of the disease. (A full account of this investigation will be published separately[14].)

Recently, we undertook a systematic examination of the organic acids in the urine from a group of patients suffering from a hereditary, progressive loss of hearing. A large family in Norway with over 80 members suffers from this unusual disorder, which results in deafness at the age of about 30–40 years[15]. No other clinical symptoms have been found. Capillary-column GC–MS demonstrated that the urine samples from these patients contain two abnormal metabolites, identified as 3-hydroxyisovaleric acid and 3-methylcrotonylglycine. These metabolites are intermediates in the metabolism of leucine, and are likely to accumulate if the enzyme 3-methylcrotonyl-CoA carboxylase is partially defective. The disorder is now under thorough investigation, using a variety of biochemical methods, enzyme determinations in fibroblast cells from the patients, oto-toxicity studies and loading experiments with leucine. Fig. 3 shows the effect of a peroral intake of 20 g of leucine on the pattern of carboxylic acid in the urine. The bottom capillary gas chromatogram is from a normal person and the top chromatogram is from one of the deaf patients. Large amounts of 3-methylcrotonylglycine and 3-hydroxyisovaleric acid have accumulated in the patient, suggesting impaired activity of the above-mentioned enzyme.

Our preliminary studies clearly show that the deaf patients have a partial block in the degradation of leucine. It remains to be proved, however, that this enzyme defect is the cause of the deafness.

Carbohydrates in seminal plasma

The causes of male infertility are frequently very difficult to establish. Although

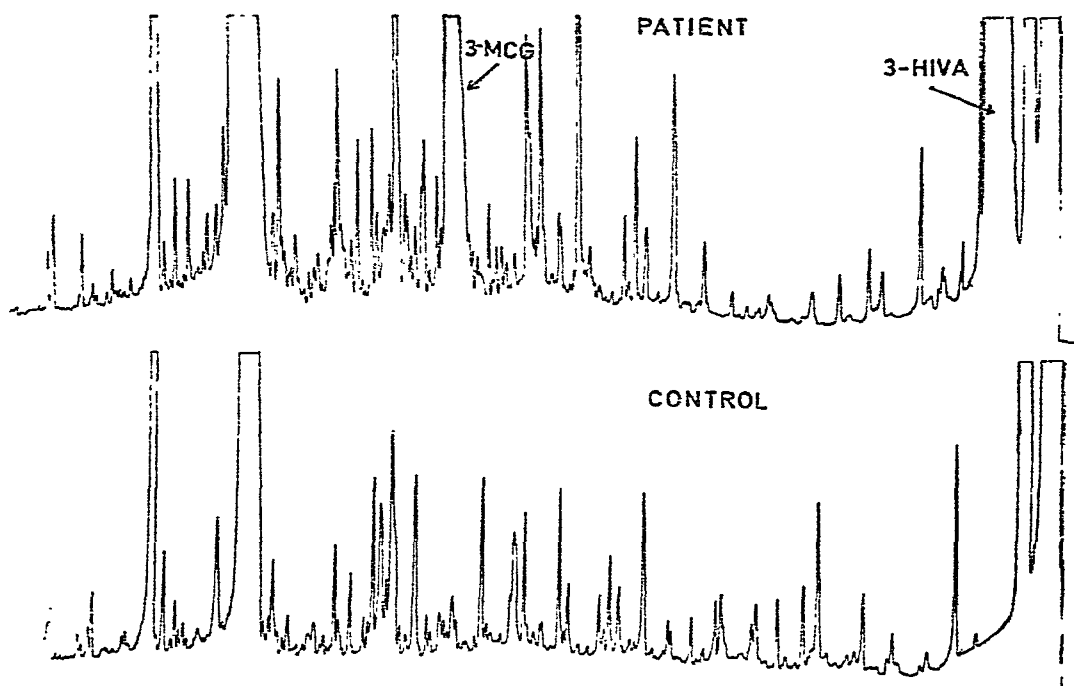

Fig. 3. Organic acids in normal urine and in the urine of a patient with hereditary progressive deafness. The normal subject and the patient were given 20 g of leucine *per os*, and urine was collected during the subsequent 3 h. Aliquots were analyzed on a 25-m SE-30 glass capillary column as described in the text. Extractions and derivatization as in Fig. 1. 3-HIVA = 3-hydroxyisovaleric acid; 3-MCG = 3-methylcrotonylglycine.

morphological methods and clinical chemical methods (*e.g.*, determination of zinc, magnesium, γ-glutamyltranspeptidase and fructose) are in use, the clinical significances of these determinations are uncertain. The latter methods also often fail to differentiate between various types of infertility. There is consequently a great need for more specific diagnostic methods. It is possible that the determination of carbohydrate profiles of seminal fluid might be such a supplementary method. This is based on the general assumption that the carbohydrate metabolism provides an important source of the energy required by the spermatozoa. Disturbances in the metabolism of sugars may therefore be a contributory factor to infertility. In this context, it should be mentioned that an example ascribing infertility (in three brothers) to a defect in carbohydrate metabolism has recently appeared[16].

The complete separation of complex carbohydrate mixtures, like that of seminal fluid, is difficult to achieve on packed GC columns. Greatly improved separation is, however, found on glass capillary columns, as demonstrated in Fig. 4, which shows the carbohydrate pattern of a normal seminal fluid. Two hitherto unrecognized components of semen were found (glycerol and erythritol), but several other components still remain unidentified. The role of variations in the carbohydrate profile in seminal fluid in healthy and diseased states are under investigation in our laboratory.

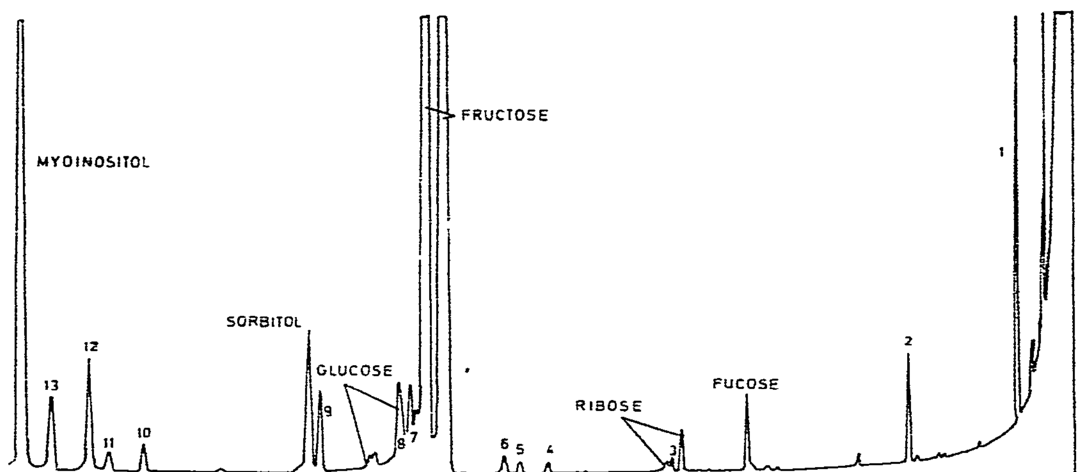

Fig. 4. Carbohydrate pattern in normal seminal fluid. Seminal fluid (0.5 ml) was filtered through a Millipore PSAC membrane and the filtrate was passed through a mixed-bed ion-exchange resin [Dowex 1 (CO_3^{2-}), Dowex 50 (H^+)]. The eluate was evaporated to dryness and derivatized with methoxylamine and BSTFA in pyridine[17]. The GC column was a 25-m SE-30 glass capillary column and the GC conditions were as described in the text. The named peaks represent previously described carbohydrates of semen. Peak 1 and 2 were identified as glycerol and erythritol. Peaks 3–13 are unknown.

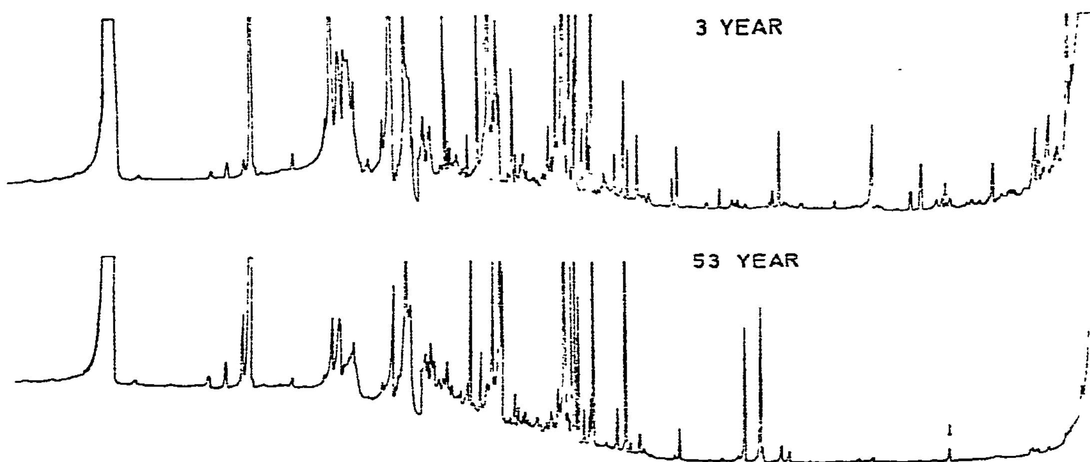

Fig. 5. Metabolic profile of unsaponifiable components of human adipose tissue. Top chromatogram is from a 3-year-old boy, bottom chromatogram from a 53-year-old man. Fat (4 g) was refluxed with sodium hydroxide (40 ml of 2 *N* NaOH in aqueous ethanol) and extracted with re-distilled diethyl ether after dilution with 60 ml of water. The ether extract was washed five times with dilute NaOH and evaporated to dryness at room temperature. The residue was re-hydrolyzed with 2 *N* NaOH (30 min at 100°) and re-extracted with diethyl ether. The extract was concentrated and injected into the capillary column (25-m, SE-30). Splitless injections were used, and the column temperature was programmed from 80° to 280° at a rate of 6°/min. Reagent blanks were run as controls.

Multicomponent analyses of human fat biopsies

During life, man is continuously exposed to a variety of chemicals, many of which may be of a hazardous, *e.g.* carcinogenic, nature. Because of the high solubility of many organic compounds in lipid material, it is likely that many environmental contaminants may accumulate in the fat deposits in the body. Thus, it is well known that pesticides, chlorinated hydrocarbons, etc.[18–20] have a great tendency to accumulate in adipose tissue. Capillary-column GC–MS–computer methods offer the possibility of investigating in detail the chemical contaminants in human fat biopsies and to estimate variations in the chemical content with age, smoking habits, occupational exposure, etc. Fig. 5 shows two capillary-column chromatograms of the non-saponifiable, alkali-stable and diethyl ether-soluble fraction of fat biopsies. The top chromatogram is from a 3-year-old child and the bottom chromatogram is from the fat deposits of a 53-year-old man. The peaks represent hydrocarbons (including polynuclear aromatics), alcohols, steroids and other substances that withstand the chemical pre-treatment described in the legend. It is striking that over 100 compounds of this chemical nature are present in human fat. There are also marked quantitative and qualitative differences between "young" and "old" adipose tissue. Further investigations are now in progress to clarify the significance of these variations.

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