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CAPILLARY COLUMN GAS CHROMATOGRAPHY–MASS SPECTROMETRY IN STUDIES ON RHEUMATOID ARTHRITIS

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SUMMARY

A method is described for determining the organic acid profile of synovial fluid. The method requires 50–100 μl of sample and involves ion exchange for isolation of the acids followed by combined capillary column gas chromatography–mass spectrometry. Normal synovial fluid and synovial fluid from patients with rheumatoid arthritis show a similar qualitative pattern of acids (mainly fatty acids) resembling the pattern found in serum. The concentrations of the organic acids in the abnormal synovial fluids are 5–10 times higher than those in the fluid from the normal joints. In patients receiving treatment with gold thiomalate, free thiomalate was excreted in the urine. The release of this thiol upon gold therapy is of consequence for the further understanding of the mechanism of action of certain drugs against rheumatoid arthritis.

INTRODUCTION

Chromatographic profiling of body fluids has proved to be of considerable importance in the diagnosis and studies of many human diseases, particularly metabolic disorders (for a review, see ref. 1). Most published work has dealt with the analyses of urine and serum, and to a lesser extent with other extracellular fluids such as cerebrospinal, amniotic, seminal and dialysis fluids[1]. The increased sensitivity of capillary over packed columns has made it possible to carry out profiling analyses of small (5 mg) tissue biopsies[2], and gas chromatographic–mass spectrometric (GC–MS) methods also open up the possibility of studying body fluids of which very limited amounts are available, *e.g.*, synovial fluid (the lubricating fluid of the joints). In this paper, a capillary column GC–MS method is described that can be used to acquire information on the organic acid profiles of synovial fluid using as little as 50 μl of material. The method has been used to analyse the organic acid content of normal

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synovial fluid and to establish the changes that occur in rheumatoid arthritis. Further, as several of the patients were undergoing treatment with gold thiomalate (Myocrisin), and because we have recently suggested that thiomalate and not only gold may be an active agent against the disease[3], GC–MS was used to search for the presence of free thiomalate in urine, serum and synovial fluids of the patients.

The results suggest that GC and MS may be valuable tools in research aimed at the further understanding of rheumatoid arthritis and its treatment with certain drugs.

EXPERIMENTAL

Chemicals

All solvents (*e.g.*, pyridine, diethyl ether) were re-distilled before use. Pyridinium acetate reagents were prepared from pyridine and glacial acetic acid. N-Nitrosomethylurea for diazomethane synthesis was obtained from K & K Labs., Plainview, N.Y., U.S.A. Diethylaminoethyl (DEAE)-Sephadex A-25 was a product of Pharmacia (Uppsala, Sweden). Myocrisin [gold(I) thiomalate] and sodium thiomalate were supplied by May & Baker (Dagenham, Great Britain). All other reagents were commercially available products of analytical-reagent grade.

Synovial fluid collection

Lidocaine was used to anaesthetize the outer region of the knee, which was subsequently approached with a needle from the medial aspect of the joint. The synovium was carefully penetrated and the highly viscous, colourless and clear liquid present in the joint was slowly withdrawn into a syringe. From the swollen, inflammatory knees of patients suffering from rheumatoid arthritis several millilitres of synovial fluid could often be collected. Normal joints, however, contain small amounts only of the lubricating fluid, and from the knees of five healthy volunteers of age 23–34 the volume of synovial fluid obtained varied from 60 to 250 μl. Blood samples were withdrawn in the usual manner simultaneously with collection of the synovial fluids.

Sample preparation for organic acid profiling

Synovial fluid or serum (50–100 μl) was diluted with 2 volumes of distilled water (used to rinse the pipette) and mixed with 4 volumes of absolute ethanol. Nonanedioic acid was added as an internal standard. The precipitate containing proteins and other high-molecular-weight compounds was removed by centrifugation. The ethanol in the supernatant was evaporated in a stream of nitrogen, and the sample was applied to a small anion-exchange column consisting of a Pasteur pipette containing 1 ml of swollen, carefully pre-washed DEAE-Sephadex A-25 in the acetate form. The column was washed with distilled water (5 ml) to remove non-acidic constituents. The retained acids were eluted with pyridinium acetate (4 ml of a 1.5 *M* solution) and lyophilized. The residue was dissolved in methanol–diethyl ether (1:1) and methylated with diazomethane. After 15 min at room temperature, the mixture was concentrated in a stream of nitrogen before injection into the GC–MS instrument fitted with a glass capillary column. For each patient the synovial fluid sample, the serum sample and a procedural blank (water + internal standard) were run together. Extreme care was taken to eliminate all sources of contamination. The ion-exchange

resin in particular would give rise to many extra GC peaks if it was not washed sufficiently well before use.

Sample preparation for thiomalate determination

Serum and synovial fluid were pre-treated (2 h at room temperature) with dithioerythritol (1 m*M*) to release any thiomalate that might be bound to proteins via mixed disulphide linkages. The proteins were then precipitated with ethanol (final concentration 80%) and removed by centrifugation. The ethanolic extract was evaporated to dryness in a stream of nitrogen, the residue was redissolved in methanol–diethyl ether (1:1), acidified with a drop of methanolic hydrochloric acid and derivatized with diazomethane. After 30 min at room temperature in the presence of excess of the methylating agent, the trimethyl derivative of thiomalate was formed quantitatively. After concentration, an aliquot was injected into the capillary column GC–MS–computer system operated in the selective ion monitoring mode.

Urine samples were acidified and extracted six times with 3 volumes of diethyl eter. This procedure extracted about 60% of the thiomalate. The solutions were combined, dried over anhydrous sodium sulphate, methylated with diazomethane and analysed as described above.

Gas chromatography–mass spectrometry

GC–MS was performed on a Varian Model 112 combined gas chromatograph–mass spectrometer (Varian-MAT, Bremen, G.F.R.) to which an on-line data system (Spectrosystem 100, Varian-MAT) with dual discs and a magnetic tape unit were attached. The gas chromatograph was a Varian Model 1400 (Varian, Walnut Creek, Calif., U.S.A.) and the glass capillary column (LKB, Stockholm, Sweden) was 25 m in length, 0.28 mm O.D. and wall-coated with SE-30. The number of theoretical plates was $80–85 \cdot 10^3$. The capillary column led directly into the ion source of the mass spectrometer, which was equipped with dual turbomolecular pumps. The flow-rate of helium carrier gas was 1 ml/min. A splitting ratio of 1:30 was normally used and the injection temperature was 230°. For the profiling analyses the column temperature was maintained at 50° for 5 min after injection, and was then increased at 6°/min to 250°. Mass spectra were recorded every 2 sec using the repetitive scanning mode.

For analysis of thiomalate the instrument was kept isothermally at 100°, and the computer-operated mass spectrometer was set to monitor the fragments of *m/z* 192, 160 and 132, which are characteristic ions in the mass spectrum of S-methyl-thiomalate dimethyl ester (see Fig. 4). This compound was eluted after *ca.* 5 min under the above conditions.

Peak identification

The major peaks of the organic acid profiles were identified by comparison with GC retention times (and retention indices calculated from running the sample in the presence of added alkane standards) and MS data for authentic compounds. Other peaks were tentatively identified by computer matching of their mass spectra against a collection of reference spectra in Oslo[4] and in Gothenburg (the Scannet file[5]).

RESULTS

Synovial fluids and sera from five healthy controls and from fifteen patients with rheumatoid arthritis were analysed. Typical organic acid profiles are shown in Figs. 1 and 2. There are striking qualitative similarities between the profiles of serum and synovial fluid from the same person, whether he is normal (Fig. 1) or a rheumatoid arthritis patient (Fig. 2). Comparison of Figs. 1 and 2 shows that the amount of almost all organic acids in normal synovial fluid is much less than that in the pathological fluids. Analyses of all fifteen rheumatoid arthritis patients pointed in this direction, *i.e.*, the concentrations of organic acids found in their synovial fluids were between 5 and 10 times higher than those in the normal subjects.

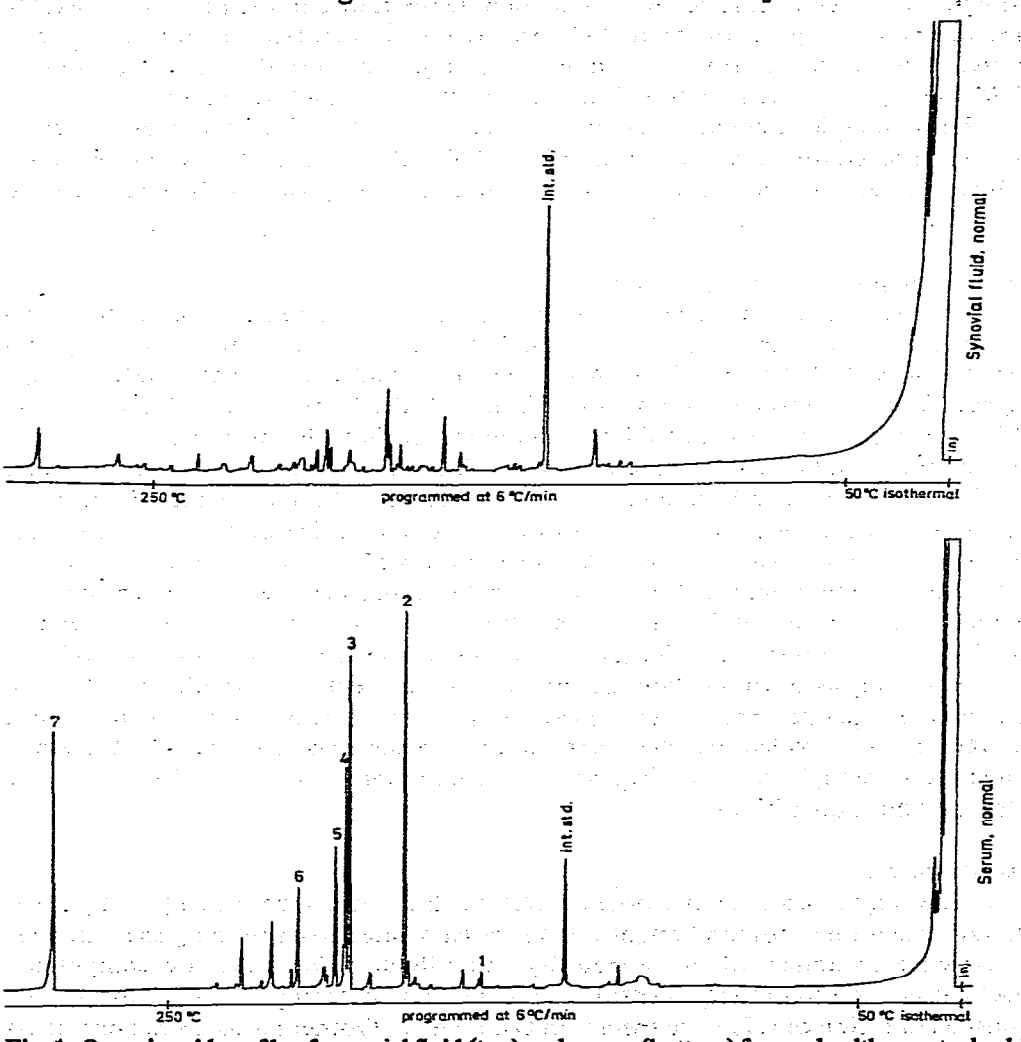

Fig. 1. Organic acid profile of synovial fluid (top) and serum (bottom) from a healthy control subject. The experimental conditions are described in the text. Peaks: 1 = myristic acid; 2 = palmitic acid; 3 = linoleic acid; 4 = oleic acid; 5 = stearic acid; 6 = arachidonic acids; 7 = cholesterol. Internal standard = nonanedioic acid

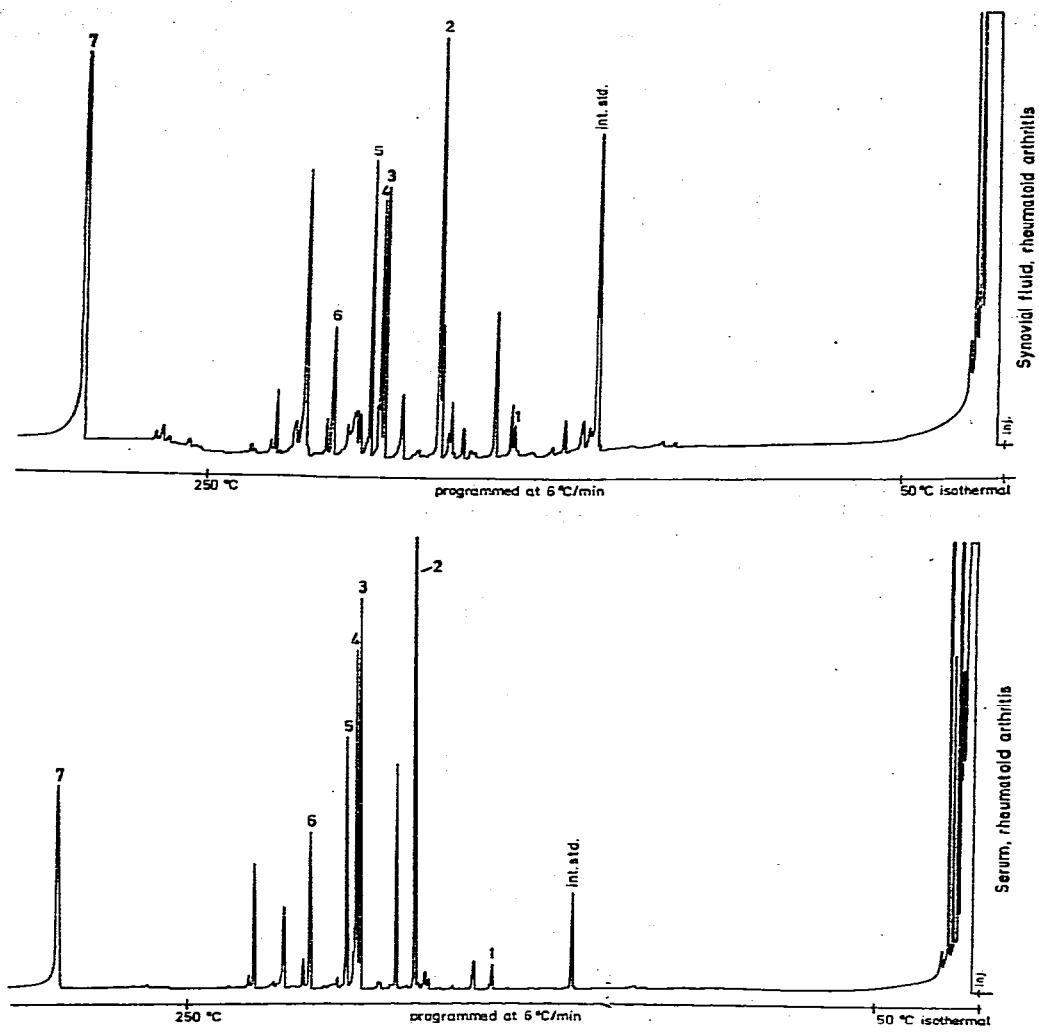

Fig. 2. Organic acid profile of synovial fluid (top) and serum (bottom) from a patient with rheumatoid arthritis. Experimental conditions and peak identifications as in Fig. 1.

The major peaks were identified as fatty acids, and these were present both in the normal and in the pathological synovial fluids. When the sensitivity of the instrument was increased, it could be seen that synovial fluids do contain a large number of organic acids, and that certain components are found in the pathological fluids that are absent in normals, and *vice versa*. No attempts were made to identify these minor and probably insignificant changes. In the synovial fluid of one patient uric acid was found as a major peak, indicating that this particular patient might also have been suffering from uric acid gout.

Fig. 3 (top) shows the mass spectrum of thiomalate (trimethyl derivative). The three characteristic ions shown were monitored in the search for free, unbound

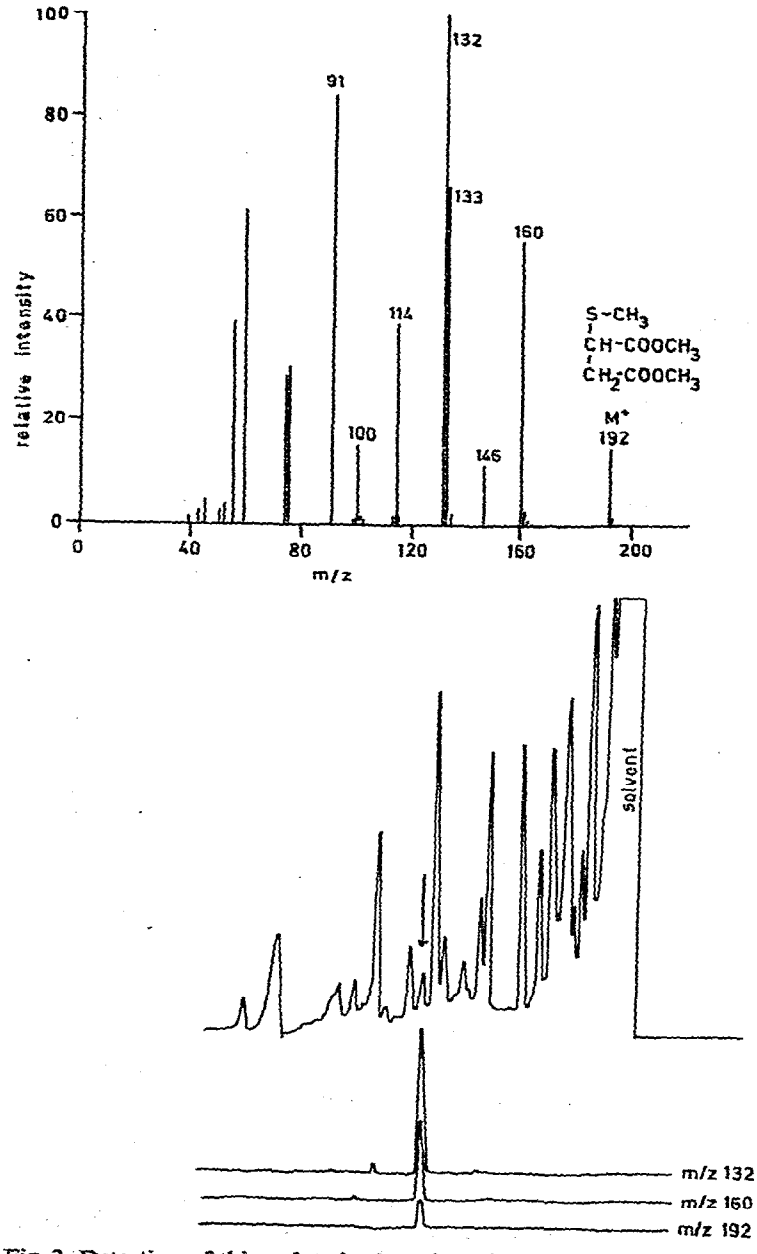

Fig. 3. Detection of thiomalate in the urine of a patient with rheumatoid arthritis. The patient had been given a standard dose (50 mg) of Myocrisin [gold (I) thiomalate] 12 h prior to collection of the urine sample. Top: mass spectrum of S-methyl thiomalate dimethyl ester. Middle and bottom: isothermal (100°) gas chromatogram of urinary organic acid methyl esters and monitoring of three characteristic ions of S-methyl thiomalate dimethyl ester. For experimental details, see text.

thiomalate in the body fluids (Fig. 3, bottom). In patients who had received Myocrisin injections 12–24 h prior to the sample collection, free thiomalate was found in the urine (see Fig. 3) but not in the serum or synovial fluid. This finding is of importance, as discussed below, in our attempts[3] to understand the mechanism of action of Myocrisin and penicillamine against rheumatoid arthritis.

DISCUSSION

Synovial fluid has not been widely studied by GC–MS. Brooks *et al.*[6] used GC and an electron-capture detector to examine synovial fluids from patients with traumatic arthritis and arthritis due to bacterial infections. In these cases, as in the case of rheumatoid arthritis, several millilitres of fluid can be obtained from the joints. If analyses of normal synovial fluid are to be included for comparative purposes, however, a sensitive micro-scale method has to be used owing to the small amount of fluid available. Such a method is described here.

Comparisons of the organic acid profiles of normal synovial fluid and of synovial fluid from patients with rheumatoid arthritis allow some conclusions to be drawn. Firstly, the organic acid pattern of both normal and pathological synovial fluids strongly resembles the profile in the serum from the same person. This supports the view held by many workers that synovial fluid probably represents some kind of ultrafiltrate of serum. Secondly, the amounts of organic acids in normal synovial fluid are low and less than the amounts found in serum. The synovial fluids from the patients, on the other hand, contain 5–10 times higher concentrations of nearly all of the organic acids, indicating a more rapid influx of serum constituents into the fluid of the diseased joint. Such a change in the chemical composition of the synovial fluid is likely to alter its lubricating efficiency, probably in a negative direction.

The finding of free thiomalate in urine, but not in serum and synovial fluids, from patients on Myocrisin treatment, corroborates results we recently obtained[7] in animal experiments using doubly isotope-labelled gold thiomalate (Au^{195}, C^{14}). It is now clear that after intramuscular injection of the latter drug there is a release of thiomalate, which is a typical thiol analogous to penicillamine, another potent drug against rheumatoid arthritis. Perhaps it is the thiomalate part of Myocrisin and not the gold (or both) which is the active component of the drug.

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