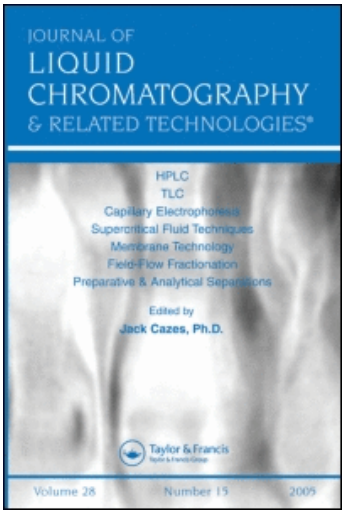

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Metabolic Profiling Using Reversed Phase High Performance Liquid Chromatography: Analysis of Urine from Patients with Rheumatoid Arthritis

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METABOLIC PROFILING USING REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY : ANALYSIS OF URINE FROM PATIENTS WITH RHEUMATOID ARTHRITIS

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ABSTRACT

Optimal conditions are described for the reversed phase HPLC separation of UV absorbing constituents including peptides and proteins in urine. Metabolic profiles have been obtained for urinary constituents in the molecular weight range <1000, 1000-10,000, and >10,000 for healthy controls, patients with rheumatoid arthritis and osteoarthritis. A computer program was employed to compare individual and groups of profiles. Considerable variation was encountered in the patterns of constituents and comparison of the metabolic profiles revealed no diagnostically significant differences between the various groups of subjects.

INTRODUCTION

The concept that individuals have a 'metabolic fingerprint' which can be defined by the amount and variety of constituents in their biological fluids arose in the late 1940's (1,2). An extension of this idea allows disease states to be identified by

their 'metabolic profile' (3). Such a system relies on an high resolution technique which allows the separation, detection and quantification of many components in one sample in one analytical run. This contrasts with 'multiphasic screening' in which there are single tests for each of the components.

The techniques most suited to analysing many components in a single run are chromatographic, especially gas chromatography (GC) and high performance liquid chromatography (HPLC). Metabolic profiling, using GC and GC/mass spectrometry has been used to identify as many as 300 substances in a single sample. Characteristic patterns have been produced for various categories of urinary metabolites such as steroids (4) and acids (5). The GC analysis of urinary acids has been used in the diagnosis of genetically determined organic acidurias, diabetes mellitus (6) and neuroblastoma (5, 7, 8, 9).

The use of high performance liquid chromatography in metabolic profiling has evolved with the development of improved equipment, much of the earlier work having been carried out using amino acid and nucleotide analysers (1). Scott (10) and co-workers (11, 12) have used such a system to detect over 140 components in urine, though analyses could take up to 40 hours per sample. Later work (10) showed a diurnal variation in urinary constituents, so that subsequent work utilized 24 hour urine collections. Recently Knudson et al., (13) have employed reversed phase HPLC to study UV absorbing components in urine from haemodialysis patients and were able to separate over 20 components.

Peptiduria and proteinuria have been demonstrated in rheumatoid patients without renal involvement and are thought to mirror connective tissue involvement (14-20). The majority of studies, however, have involved measurement of individual peptides or proteins and few studies have examined the pattern of excretion of urinary peptides/proteins or assessed their diagnostic significance in patients with rheumatoid arthritis.

The objectives of this study were (i) to optimise the chromatographic conditions for the reversed phase HPLC separation of UV absorbing constituents including peptides and proteins in urine and (ii) to apply these in the HPLC analysis of urines from patients with rheumatoid arthritis, and to assess whether such metabolic profiling would be of diagnostic use.

MATERIALS AND METHODS

Clinical Material

Urines (24 hour collections using azide as preservative) were obtained from healthy controls (ten), patients suffering from rheumatoid arthritis (eleven) and patients suffering from osteoarthritis (eight). Those suffering from rheumatoid arthritis were either housebound and contacted through their general practitioner or had been admitted to hospital for assessment and treatment. Those with osteoarthritis were all hospital in-patients. Patients suffering from both rheumatoid and osteoarthritis (two) were also studied. Detailed medical and drug histories and, where possible, results of any relevant investigations were obtained from the patient's

medical notes. Informed consent was obtained from each subject. The osteoarthritis patients acted as an ill control group. They had a similar age and sex distribution, and degree of mobility to the rheumatoid patients.

Urine volume and creatinine were measured, the latter using the Jaffé reaction on a Technicon AAI AutoAnalyzer system (21). No urine was used which had a creatinine level of less then mean $\pm$2 standard deviations for the relevant subject group in order to ensure complete 24 hour collection. Urines were screened for protein, haemoglobin, ketones, glucose and pH using Labstix (Ames Co., Slough) to exclude other pathologies. Samples were stored frozen at -4^{o}C until analysed.

Fractionation and concentration of urines

Urine contains a large number of UV absorbing components in a complex matrix. In order to simplify the analytical problem urine was fractionated into three molecular weight fractions corresponding to >1000, 1000-10,000, and <10,000 Daltons.

Preliminary studies indicated that fractionation of the urine prolonged column life and improved resolution. Urines to be analysed were, therefore, first fractionated by ultrafiltration using a stirred flow cell (Chem Lab Instruments Ltd., Essex) and Amicon ultrafiltration membranes (Amicon Ltd., Surrey). An aliquot of urine (20 ml) to be fractionated was first vacuum filtered through a Whatman No. 42 filter paper to remove particulate matter and then at a pressure of 60 psi, the sample was ultrafiltered through a UM10 membrane (Amicon Ltd.,) with a molecular weight cut-off of 10,000 until 2 ml remained above the

membrane. Distilled water (20 ml) was then added and the ultrafiltration continued until approximately 2 ml remained above the membrane. This was then retained as the fraction with molecular weight greater than 10,000. The combined sample and wash water which had passed through the membrane was then ultrafiltered using a UM2 membrane (molecular weight cut-off of 1,000). When 2 ml remained above the membrane this was then washed through with distilled water (20 ml) until approximately 2 ml remained above the membrane. Both fractions, that above the membrane (molecular weight 10,000-1,000) and through the membrane (molecular weight less than 1,000) were retained. Samples were stored frozen at -4°C.

High performance liquid chromatography

Optimisation of chromatographic conditions

HPLC chromatographic conditions were optimised to yield the maximum number of peaks in an analysis time of less than 2 hours. Thus the type and concentration of the organic portion of the mobile phase, and the concentration and pH of the buffer in the mobile phase were each optimised in turn.

Analysis

Urines were analysed at ambient temperature by high performance liquid chromatography using a Pye Unicam LC3 (Pye Unicam Ltd., Cambridge) with variable wavelength UV detector and valve injection. The following conditions were used; Shandon Hypersil- ODS column (25 cm x 5mm, id, Shandon Southern, Runcorn) packed with a Stansted Fluid Power pump (Magnus Scientific, Sandbach) at 4,000 psi,

degassed mobile phase of 75% 0.01 mol/l potassium dihydrogen phosphate, 24.9% methanol (HPLC grade, Rathburn Chemicals, Walkerburn) and 0.1% orthophosphoric acid (Fisons Ltd., Loughborough) at a flow rate of 0.6 ml/min. A sample size of 10 µl was injected. The absorbance at 210 nm was measured and the signal from the detector was taken to a chart recorder and also (amplified x 10) to a data logger.

Data Handling

Data was logged every second from the beginning to the end of the HPLC run and at the end of each run a digital printout of each reading, and the same information on paper tape, were obtained. The printout was used simply to check that data had been registered. The paper tape produced was then entered into a Data General Nova 2 computer and the HPLC trace displayed on a storage oscilloscope. The trace was then checked against the original chart recorder output to ensure that all peaks had been entered and that none had been added. A second computer program (22) was then run to produce (a) a 'stick' diagram of peaks at the calculated retention times and peak height expressed in absorbance, (b) a listing of retention times at which peaks occurred, with the peak height in absorbance units (corrected to a constant urine volume to take into account the final volume of the ultrafiltered urine fractions) and (c) a listing of retention times at which peaks occurred with the peak height in absorbance units corrected for urine creatinine concentration.

Statistical analysis

The precision of measuring the retention time of the first peak was found to give a coefficient of variation of 4%. Allowing for this, the number of peaks occurring at a given retention time, e.g., 4.0 - 4.5 minutes in a run for a given urine fraction and within each group was noted. These data were then analysed by the Chi Squared Test for three groups.

RESULTS

The optimisation of chromatographic conditions led to the use of the HPLC conditions described in the Methods section.

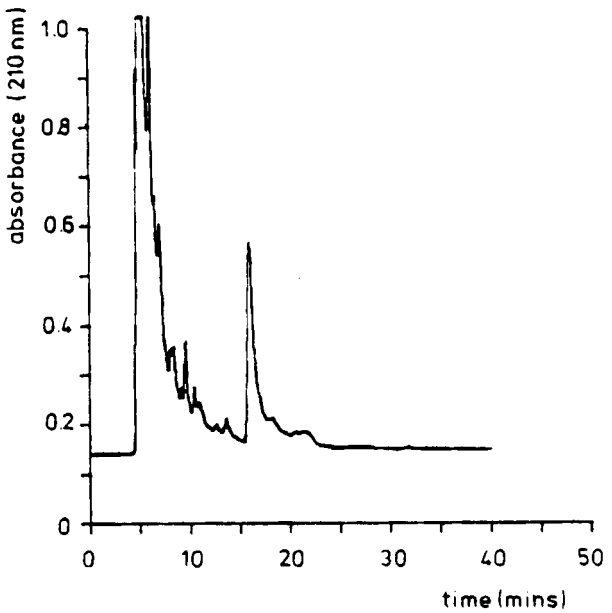
No abnormal results were obtained on testing the urines from the three groups of subjects with Labstix.

A total of 31 subjects was investigated, and since each urine was divided into three molecular weight fractions, 93 HPLC analyses were made. To illustrate these results typical HPLC runs, and stick diagrams, for a healthy subject, a patient with rheumatoid arthritis and a patient with osteoarthritis are shown in Figures 1, 2, and 3, respectively.

Visual inspection of the stick diagrams corrected for either urine volume or creatinine concentration from the same fractions within a disease group showed variation in the number of peaks and in the peak heights. This was true for each fraction of each patient group. No obvious differences could be found between the urine fractions from each disease group; normal healthy controls, rheumatoid arthritis patients and osteoarthritis patients.

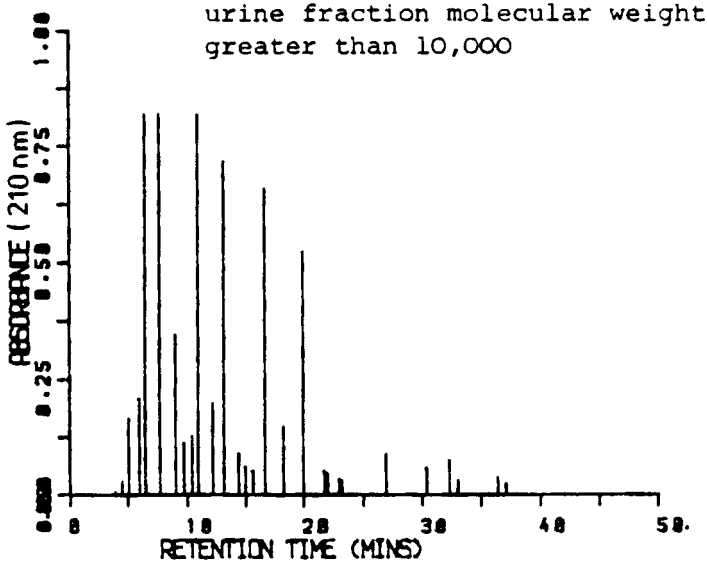
a

healthy subject



c

urine fraction molecular weight greater than 10,000



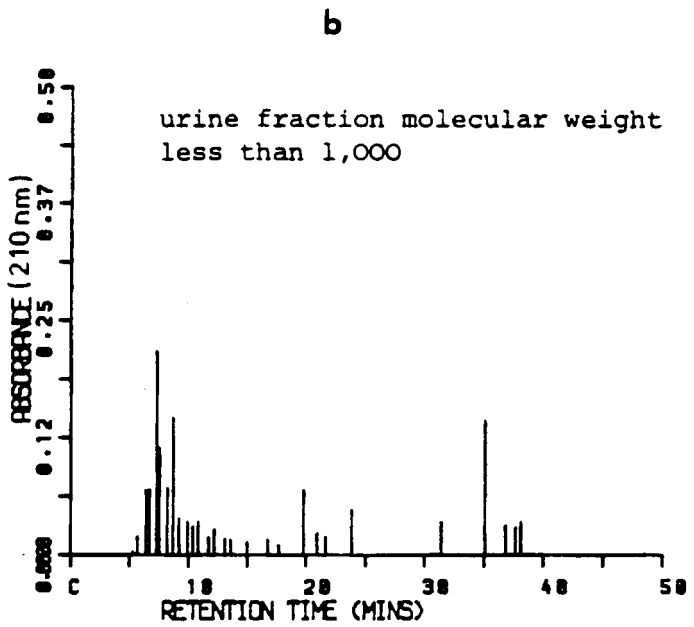


Figure 1. HPLC trace and stick diagrams of urine UV absorbing constituents from a healthy subject in the molecular weight range

(a) 1,000 - 10,000 (b) less than 1,000 and (c) greater than 10,000

In view of the complexity of the 'stick diagrams' a more rigorous statistical analysis of the data was, therefore, carried out to assess whether there were any significant differences in the HPLC chromatograms of each urine fraction of the disease groups. This was performed on the presence or absence of peak basis, thus ignoring peak height which showed great variation both within and between groups. The Chi Squared Test, however, revealed no peaks which might be present or absent in one particular group only, and no differences could be demonstrated between HPLC metabolic profiles of the three fractions of the three groups of subjects.

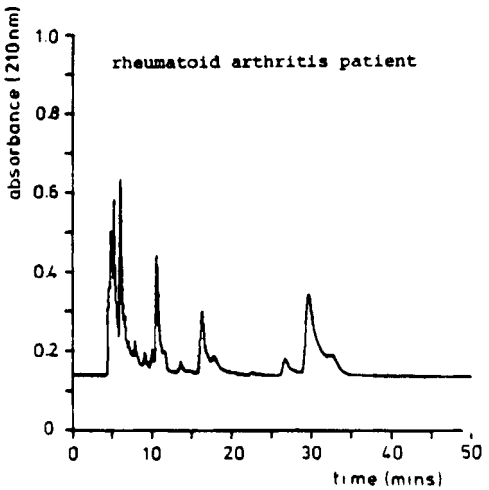


Figure 2. HPLC trace of urine UV absorbing constituents in the molecular weight range 1,000 - 10,000 from a patient with rheumatoid arthritis.

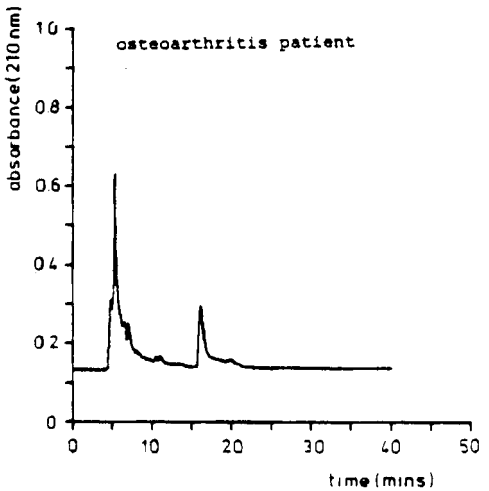


Figure 3. HPLC trace of urine UV absorbing constituents in the molecular weight range 1,000 - 10,000 from a patient with osteoarthritis.

DISCUSSION

Several problems arise when generating and interpreting the metabolic profiles produced by a technique such as HPLC and earlier attempts of data handling and comparison have not been entirely successful. The main difficulty is in the comparison of a large number of HPLC chromatograms. Many studies have either used pooled urine samples or have used only a small number of subjects when comparing metabolic profiles from disease groups with those from a normal healthy group. Such approaches have obvious disadvantages. If a large number of subjects is to be assessed, as in this study, some method of data reduction and comparison is necessary. The first step is to convert the UV detector output into a digital form and from this identify each peak in each run, and then to measure peak height and retention time. Using these data a large number of HPLC runs can be compared. The problem of identifying peaks in digital data is a complex one. In many cases, where resolution is high and only a few peaks are of interest, commercial integrators will suffice. However, with more complex patterns, more flexibility is required. Whilst several computer programs, generally of great mathematical complexity, have been written, most attempt the following:- (a) to recognise the beginning and end of a peak, (b) to locate the peak maximum, (c) to locate the inflection points, (d) to calculate the peaks area, (e) to resolve overlapping peaks, (f) to maintain an accurate baseline and (g) to distinguish peaks from noise (23, 24). In order to achieve this, calculation of

the second derivative is necessary and certain assumptions regarding peak shapes of overlapping peaks and their relative contributions have to be made. In this study a less complex approach was taken, noise was allowed for, peaks were identified by locating increases or decreases in absorbance over or below certain empirically determined values, and peak height, rather than peak area, was measured. Measurement of peak height meant that no attempt at assessing the relative sizes and shapes of incompletely resolved peaks was necessary.

Visual inspection of a large number of chromatograms for comparative purposes is impossible and the data handling procedures presented here have facilitated the comparison of HPLC runs. Visual and statistical comparison revealed considerable variation in the pattern, even within one group, and no significant differences have been shown in HPLC metabolic profiles of the three fractions of the three groups studied.

One of the difficulties in metabolic profiling of complex biological fluids composed of an unknown number of constituents is that it is impossible to assess when complete resolution of all the constituents has been achieved. It is possible that several different analytical methods might give what would appear to be adequate resolution of constituents, but not all methods would resolve the clinically important substances.

Most previous metabolic profiling studies based on HPLC have used ion-exchange supports. Metabolic profiling using a reversed

phase support described in this study has several advantages, namely the support is relatively inexpensive, is stable and does not have to be regenerated after each run.

CONCLUSION

This study has demonstrated the use of reversed phase HPLC as a method of metabolic profiling and has developed methods to allow the comparison of large numbers of HPLC chromatograms. No diagnostically significant differences were shown between the metabolic profiles of the three groups; rheumatoid arthritis, osteoarthritis and normal subjects. This may be due to the great variability in urinary constituents within the groups, it may reflect inadequacies in the analytical method or the fact that no differences exist.

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