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COMPARISON OF URINARY EXCRETION OF UV-ABSORBING CONSTITUENTS IN HEALTHY SUBJECTS AND PATIENTS WITH RHEUMATOID ARTHRITIS USING ANALYTICAL ISOTACHOPHORESIS

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SUMMARY

Analytical isotachophoresis has been applied to the separation of urinary constituents in healthy controls and patients with rheumatoid and osteoarthritis. Various methods of comparing isotachograms have been investigated. Significant differences have been demonstrated between the pattern of UV-absorbing components in patients with rheumatoid arthritis and healthy subjects.

INTRODUCTION

Isotachophoresis, where ions are separated on the basis of net mobility [1], has been widely used in the analysis of a variety of complex peptide and protein mixtures. Thus, analytical isotachophoresis has been employed for the quality control of peptide formulations [2, 3], in the analysis and isolation of peptides in the "middle molecule" fraction of uraemic body fluids [4, 5], and in the analysis of human serum, cerebrospinal fluid, urine and sweat proteins [6—10], enzymes [11, 12], tissue proteins [6] and soluble immune complexes [6, 13]. Conventional methods for analysing urine for UV-absorbing constituents, including proteins and peptides, are usually slow, insensitive and have a low resolving power. Isotachophoresis might be a better technique for studying these constituents. Proteinuria and peptiduria have been demonstrated in rheumatoid patients without renal involvement and are thought to mirror connective tissue involvement. Most studies have concentrated on particular proteins [14—20]. The pattern of excretion of urinary peptides and proteins in rheumatoid patients, and their diagnostic significance have not been investigated. The objective of this study was, therefore, to investigate the use of isotachophoresis in urine metabolic profiling and, in particular, to compare the pattern of excretion of constituents in the urine of patients with rheumatoid arthritis and in

healthy controls. A secondary objective was to investigate the use of various methods of data handling and pattern analysis for large numbers of samples.

EXPERIMENTAL

Clinical material

Urine samples (24-h collections using sodium azide as preservative) were obtained from ten healthy controls, ten patients suffering from rheumatoid arthritis, and six patients with osteoarthritis. Those suffering from rheumatoid arthritis were either housebound and contacted through their general practitioners, or had been admitted to hospital for assessment and treatment. Those with osteoarthritis were all hospital in-patients and acted as an "ill" control group. Informed consent, detailed medical and drug histories, and where possible results of any relevant investigations were obtained. Samples were stored frozen at −4° until analysed.

Controls

Creatinine levels of the 24-h urines were determined by the Jaffe reaction using a Technicon AutoAnalyzer II [21]. In order to ensure that complete 24-h collections had been obtained, no urine was used which had a creatinine level outside the mean ± 2 S.D. for the relevant subject group. Urines were tested for protein, haemoglobin, ketones, glucose and pH using Labstix (Ames Co.) in order to eliminate patients with renal involvement or other pathologies.

Analytical isotachophoresis

Urine samples to be analysed were first filtered through Millipore filters (0.45 μm, Millipore, London, Great Britain) to remove particulate matter. Then aliquots (10 μl) of an equivolume mixture of urine and aqueous Ampholine solution (2% v/v Ampholines, pH 3.5—10; LKB, Croydon, Great Britain) were analysed on the LKB Tachophor 2127. The leading electrolyte was hydrochloric acid (10 mmol/l) in methyl cellulose (2 g/l, high substitution; BDH, Poole, Great Britain) buffered to pH 8.5 with ammediol (2-amino-2-methyl-1,3-propanediol; Sigma, Poole, Great Britain). The terminating electrolyte was glycine (20 mmol/l; BDH) brought to pH 10.0 by the addition of barium hydroxide solid (BDH), and was filtered to remove particulate matter. The initial isotachophoresis run on the LKB Tachophor was at 150 μA until 10 kV was reached and then the current was reduced to 100 μA. A 40-cm capillary was used. Detection was by means of a UV detector (254 nm) connected to a chart recorder.

Data handling

The UV traces for each analytical run were converted to a numerical form (digitised) in the following manner. The UV trace was retraced using a chart recorder at constant speed with a variable resistor to alter the pen setting. The variable output from this retracing was fed to a data logger and readings were taken by the logger at intervals of the equivalent of one per second of the original run. From the data logger a paper tape of the digitised UV trace was then obtained.

The paper tape produced from the chart recording, i.e. the UV trace in numerical form, was then entered into a Data General Nova 2 computer and the UV isotachophoresis trace displayed on the storage oscilloscope. This 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 designed to locate peaks in the digitised data, i.e. locate and measure the maxima in the peaks, was then run to produce (a) a "stick" diagram of peaks in the sequence in which they occurred in the run, and (b) a listing of the peak heights in the sequence in which they occurred in the run, with the peak height in absorbance units.

RESULTS

Typical isotachophoresis runs and "stick" diagrams from normal, rheumatoid arthritic, and osteoarthritic subjects are shown in Fig. 1. Up to fifty different UV-absorbing components were resolved in each of the urines. Whilst there was some variation in the isotachophoresis peaks in healthy controls, this was not great. No abnormal results were found on testing the urines with Labstix. The data obtained from each run were analysed statistically in three ways (Table I).

(1) In each analytical run, in all subject groups three distinct regions or groups of peaks could be recognised in the UV trace. The number of peaks in each region and the length of that region were measured and the area under all the peaks in each region was determined by cutting out and weighing. When comparing the results from the rheumatoid arthritic subjects with those from the normal controls, the most significant differences were found using the area/number of peaks in region II. The most significantly different parameter when comparing the results from the osteoarthritic subjects with the normals was the length/number of peaks in region III.

(2) When the sequences of UV peak heights in the isotachophoresis traces from patients with rheumatoid arthritis were combined with those from the normal subjects a master sequence was obtained. Then for each analytical run the sequence of peak heights was compared with the master sequence and the presence or absence of the peaks noted. Thus, for each disease or control group of subjects the frequency of the presence of each peak was found. Using the χ^2 test the significance of the differences in the presence or absence of each peak was analysed. The χ^2 values for approximately six peaks indicated that these peaks differed significantly in their presence in the two groups. However, because of the small number of runs, the total χ^2 value, i.e. the sum of all the χ^2 values, was not significantly different. Those peaks whose presence was shown to be significantly different in the two groups were then located in each analytical run.

(3) Using the listing of the peak heights in the sequence in which they occurred in the run, for each analytical run, each peak height was compared with the next. Thus, if the second of two adjacent peaks had a larger peak height than the first, this was regarded as an increase (+), and if the peak height of the second peak was smaller this was recorded as a decrease (−). This was attempted in order to define the overall shape of the UV isotachophoresis trace.

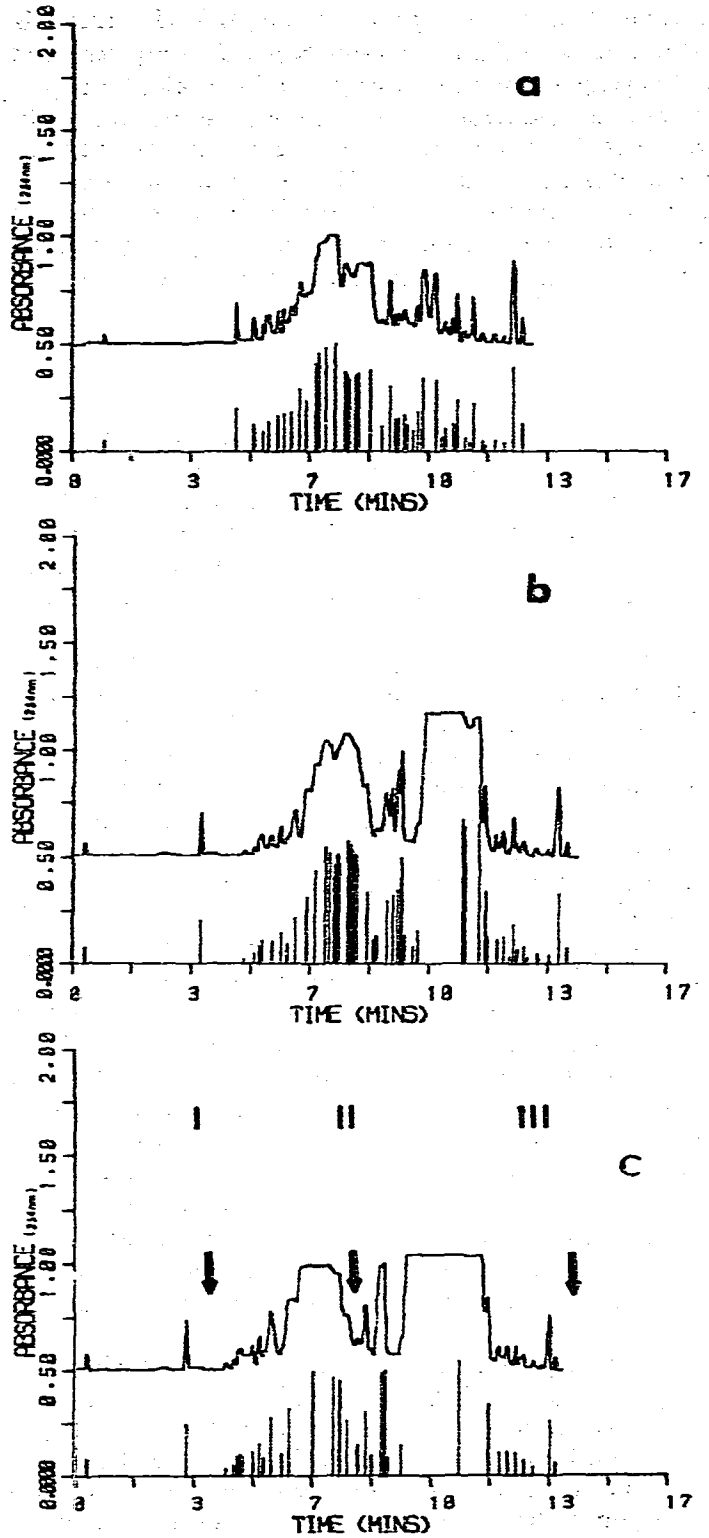
a
b
c
I
II
III
ABSORBANCE
TIME (MINS)
0
3
7
10
13
17
0.50
1.00
1.50
2.00

TABLE I

SIGNIFICANCE OF THE DIFFERENCES IN SELECTED VARIABLES IN ISOTACHOPHORETIC RUNS FROM RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS URINES COMPARED TO NORMAL, HEALTHY CONTROLS (*t*-TEST)

Variable	Rheumatoid arthritis	Osteoarthritis
	P	*P*
Total length of run	0.1	0.1
Total number of peaks	0.1	0.02
Length of region		
I	0.05	0.1
II	0.1	0.1
III	0.1	0.1
Number of peaks in region		
I	0.1	0.1
II	0.1	0.05
III	0.1	0.1
Total length/total number of peaks	0.1	0.1
Length/number of peaks		
I	0.02	0.1
II	0.1	0.1
III	0.05	0.001
Area of region		
I	0.02	0.1
II	0.1	0.1
III	0.1	0.05
Area of region/number of peaks		
I	0.02	0.1
II	0.01	0.1
III	0.1	0.01

The total numbers of increases and decreases were noted for each analytical run for each subject group (Fig. 2). In some cases a series of increases (rises) or a series of decreases (falls) occurred together in a run; these were also noted (Fig. 3). These series were compared using the Kruskal Wallis Test [22] which showed no significant differences between the groups (Figs. 2 and 3).

Fig. 1. UV traces and "stick" diagrams from the isotachophoretic analysis of urines from (a) a healthy control, (b) a patient with rheumatoid arthritis, and (c) a patient with osteoarthritis. Arrows indicate the limits of regions I, II and III.

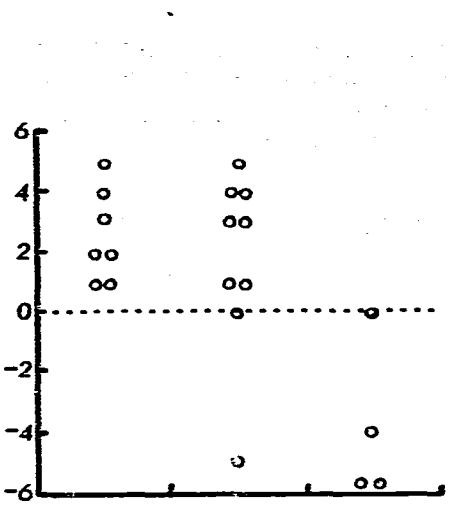

Fig. 2. Isotachophoresis: total number of increases (+) and decreases (−) in peak heights. N = Normal subjects; RA = rheumatoid arthritic patients; OA = osteoarthritic patients.

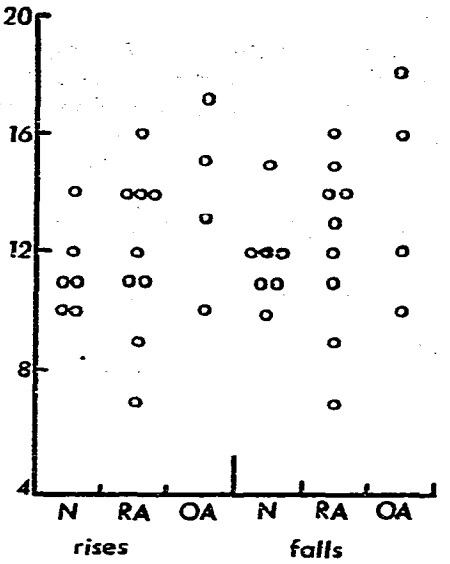

Fig. 3. Isotachophoresis: number of sequences of rises and falls in peak heights. N = Normal subjects; RA = rheumatoid arthritic patients; OA = osteoarthritic patients.

DISCUSSION

This study has shown that isotachophoresis is a high-resolution technique capable of separating urine into as many as fifty UV-absorbing components. Differences in the patterns of urine UV-absorbing constituents separated by isotachophoresis have been found between rheumatoid arthritic and osteoarthritic subjects as compared with normal, healthy controls. Six of the components showed significant differences between rheumatoid and normal subjects, but only one of these components was identified.

Comparison of UV traces from isotachophoretic runs is problematic since the location of a component on a trace is dependent upon the number and the concentration of other components present in the mixture being analysed. The presence of extra components will cause an expansion, and the absence of components a contraction of the trace.

If a large number of complex patterns from isotachophoretic runs are to be compared, which is necessary in order to overcome the problems of biological variation, some form of data handling is necessary. In this case, by digitising the data, locating the peaks in these data and measuring peak height, some data reduction was possible. Several statistical techniques were assessed in an attempt to define and compare the shapes of the UV traces. For small numbers of runs this is easily done by eye, but with a large number of runs with many peaks this becomes impossible. Whilst all three approaches tried in this study demonstrated differences between some of the UV-absorbing constituents of the subject groups, their main use was to indicate areas in the isotachophoretic run which differed between the groups. Further studies would be necessary to iso-

late and identify the constituents in these areas. More specific assays could then be developed to assess the value of measuring such constituents in the diagnosis and assessment of rheumatoid arthritis.

CONCLUSIONS

The use of isotachophoresis in the analysis of complex mixtures has, in the past, been hampered by problems inherent in the interpretation and comparison of results. The relatively simple data handling techniques described here have facilitated the comparison of UV traces from isotachophoretic runs and demonstrated significant differences in the pattern of urine UV-absorbing substances between patients with rheumatoid and osteoarthritis and healthy controls. It is envisaged that further refinement of the data handling techniques to include both UV and thermal traces will greatly enhance the usefulness of analytical isotachophoresis in metabolic profiling.

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