Journal of Chromatography, 181 (1980) 311—318
Biomedical Applications

CHROMBIO. 500

HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY OF ULTRAVIOLET-ABSORBING CONSTITUENTS OF HUMAN URINE

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(First received August 14th, 1979; revised manuscript received November 12th, 1979)

SUMMARY

A 100-μl urine sample was chromatographed on a column packed with a strongly basic macroreticular anion-exchange resin (Diaion CDR-10, 5—7 μm diameter with a nominal 35% cross linkage). The elution was performed with a linear acetate gradient from 0 to 6.0 *M* at an average flow-rate of 0.72 ml/min and at an average pressure of 104 kg/cm². The relative standard deviation of retention times and peak height was ± 4% or less. The properties of the macroreticular anion-exchange resin, the effect of the particle size, the pH of acetate buffers, and the effect of the flow-rate of the eluent on the separation were investigated. Thirty three components of urine were then resolved and named.

INTRODUCTION

Body fluids, such as urine and blood, contain hundreds of components, which have a positive correlation to pathological states [1, 2]. Scott and co-workers [3—8] developed an analytical system which separates the UV-absorbing components of human urine in less than 40 h. The microreticular

anion-exchange resin system, however, required a relatively long separation time [9—12], resulting in a low sample throughput. Thus several operational options were investigated in an effort to decrease the analysis time [9, 10, 13]. Therefore, we have studied a new anion-exchange chromatographic system using a macroreticular anion-exchange resin, which separates the UV-absorbing components of human urine in less than 120 min and with high resolution [14].

EXPERIMENTAL

Chemicals

Analytical grade ammonium acetate and acetic acid were purchased from Wako (Tokyo, Japan). The reference compounds creatine, creatinine, pyridoxine, uracil, histidine, theobromine, nicotinamide, hypoxanthine, adenosine, phenylalanine, xanthine, caffeine, tyrosine, tryptophan, theophylline, urocanic acid, uric acid, nicotinic acid, 4-aminohippuric acid, 4-hydrocy-3-methoxymandelic acid, 4-hydroxy-3-methoxyphenylacetic acid, *p*-aminobenzoic acid, hippuric acid, quinaldic acid, *p*-hydroxyphenylacetic acid, vanillic acid, kynurenic acid, *p*-hydroxyphenylpyruvic acid, benzoic acid, *p*-hydroxybenzoic acid, 3-hydroxyanthranilic acid, indoleacetic acid, and indoleacrylic acid, were also purchased from Wako.

Resin

The strongly basic macroreticular anion-exchange resins, Diaion CDR-10, with a particle size distribution of (A) 6 ± 1; (B) 11 ± 2 and (C) 25 ± 4 μm, were obtained from Mitsubishi (Tokyo, Japan).

Apparatus

A Hitachi Model 634 high-performance liquid chromatograph (Hitachi, Tokyo, Japan) was used for the urine analysis, and was coupled to a variable wavelength photometer and a 10-mV data recorder.

Column

A stainless-steel column (50 × 0.4 cm I.D.) was dynamically packed with Diaion CDR-10 at 150 kg/cm^2 using a high-pressure pump as described by Scott and Lee [15]. A slurry of the resin and water (50:50) was used for the rigid packing and then 6.0 *M* acetate buffer (pH 4.4) was run through the column for 1 h.

Sample preparation

Urine samples, usually 24-h collections, were refrigerated until complete, then frozen and stored at −20°. Before analysis, the sample was defrosted and passed through a 0.22-μm Millipore filter to remove particular matter.

High-performance anion-exchange chromatography

A 100-μl urine sample was introduced onto the column and eluted using a linear acetate gradient from 0 to 6.0 *M* at a flow-rate of 0.72 ml/min. The linear acetate gradient was formed by placing 30 ml of water in the first

chamber, and 30 ml of 6.0 *M* ammonium acetate buffer, pH 4.4, in the second chamber. During the first 25 min, the column temperature was increased from 22 to 60°, and then maintained at 60° until the end of the run. With the elevation of the temperature, the column inlet pressure dropped from 104 to 70 kg/cm^2, and with the increase of buffer concentration, the inlet pressure was raised from 70 to 135 kg/cm^2 and was maintained at 135 kg/cm^2 until the end of the run. After analysis, the column was washed with distilled water for 30 min at 150 kg/cm^2. The column was ready for the next sample when the pH of the eluate was tested to be neutral.

Assignment of the peaks

Peak assignments were carried out in three ways: firstly, by comparing the retention times of the peaks to those of known compounds; secondly, by injecting known compounds together with the samples; and thirdly, by measuring the UV spectrum with stopped-flow scanning spectrophotometry.

RESULTS AND DISCUSSION

Effects of particle size of macroreticular anion-exchange resins on the separation

A typical chromatogram of urine from a male subject is shown in Fig. 1. The column used was packed with Diaion CDR-10 having a particle size distribution of 5—7 μm (A). Other chromatograms of the same urine sample obtained when the columns were packed with resin of different particle size (B, 11 ± 2 and C, 25 ± 4 μm) were compared with Fig. 1. When these chromatograms were compared it was evident that the smaller the particle size of the resin the

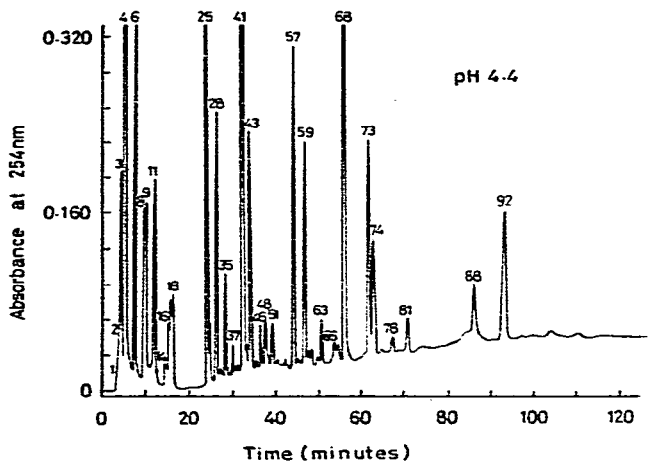

Fig. 1. A 120-min chromatogram of human urine from a normal male subject. Conditions: column, stainless steel (50 cm × 0.4 cm I.D.) packed with Diaion CDR-10; urine sample, 100 μl of a 24-h collection from a 60-kg man; temperature, increasing from 22 to 60° for the first 25 min, maintained at 60° until the end of the run; eluent, acetate buffer, pH 4.4, varying in concentration from 0 to 6.0 *M* by linear gradient, average flow-rate, 0.72 ml/min; average pressure, 104 kg/cm^2.

better the resolution. Thus, higher speed and resolution of the UV-absorbing components of human urine are dependent upon the use of resin particles of smaller size and narrower distribution.

The separation system used by Scott and Lee [9] was performed by using a longer column and high pressures; as the microreticular anion-exchange resin (12—15 μm diameter, Aminex A-27) gave severe flow-resistance and thus a limited flow-rate, even when high pressures were applied, this accordingly gave relatively longer run times. Then several operational devices, such as the use of coupled columns of anion-exchange resin followed by cation-exchange resin [9] or specific gradient programs [10], were attempted in an effort to increase the rate of sampling, but with very limited success.

Effects of the pH of acetate buffers on the separation

Chromatograms of the urine sample at different pH values of the buffer are shown in Fig. 2. Each separation was performed similar to that described above, using 6.0 *M* ammonium acetate buffer at pH 3.9, 4.4, and 4.9. At pH

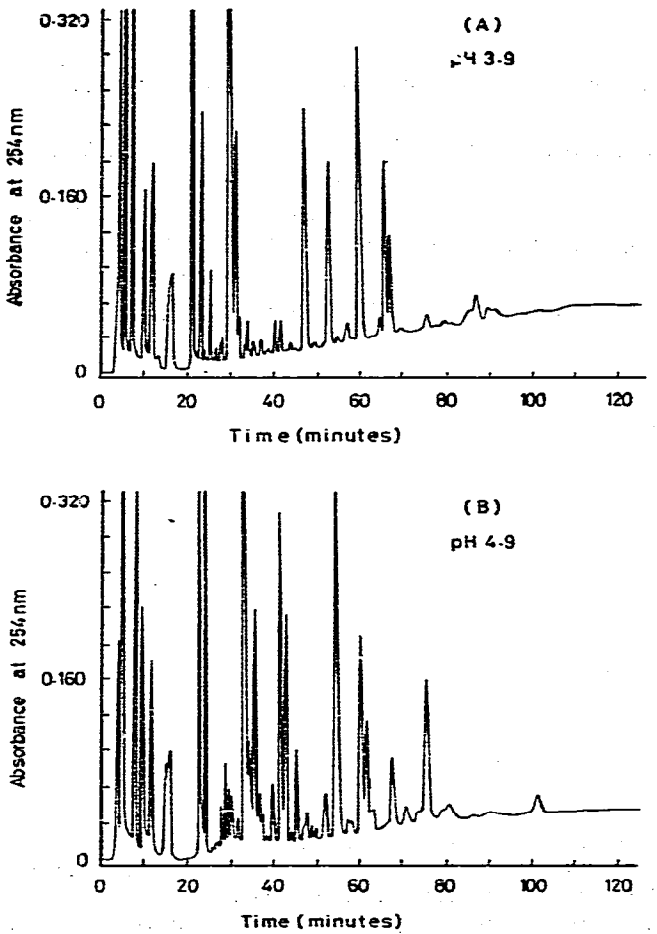

Fig. 2. Effect of the pH of the acetate buffer on the separation. Conditions are the same as in Fig. 1, except: (A) 6.0 *M* acetate buffer, pH 3.9; (B) 6.0 *M* acetate buffer, pH 4.9.

3.9 the chromatographic run required a longer elution time than at pH 4.4, and the resolution and elution after 70 min were very poor. On the other hand, the run time at pH 4.9 was shorter than at pH 4.4, but the resolution around 40 min elution was not satisfactory. Thus, the separation at pH 4.4 was used for the standard elution conditions, but the detection and estimation of a specific substance could be performed at pH 3.9 or pH 4.9.

Effects of flow-rate of eluent on the separation

Decreasing flow-rate of the eluent contributed to higher resolution, but resulted in a longer analysis time. In a run time of 120 min the number of detectable peaks was about 100, and in 200 min it was about 110, and when further increased to 12 h, 130 peaks. However, for the standard analytical system, we still adopted the 120-min system, since 100 peaks in 120 min is time-saving and more useful for practical purposes when compared with 110 peaks in 200 min or 130 peaks in 720 min.

Reproducibility of the separation of the standard separation system

To demonstrate the reproducibility of the standard separation system, quadruple chromatographic analyses were performed on the same urine sample. The elution times of eight of the major peaks were determined and were found to have a relative standard deviation of 0.81—1.08% (Table I). Thus, elution time is useful for the qualitative analysis of a substance. The peak heights of eight of the major peaks were determined and found to have a relative standard deviation of 1.04—3.55%. This peak height could be useful for quantitative analysis.

Assignment of the urine components in the chromatogram

The peaks that have been assigned so far are listed in Table II. Peak assignments were carried out by the use of reference compounds as described in Experimental. A chromatogram of uric acid and hippuric acid and their UV spectra are shown in Fig. 3. The compounds in Table II are listed in order of their elution from the separation system. These results indicate the good correlation between elution position and chemical structure. This correlation is

TABLE I

REPRODUCIBILITY OF THE SEPARATION OF HUMAN URINE

Means ± relative standard deviations of 4 determinations.

No.	Peak number in Fig. 1	Elution time (min)	Relative standard deviation (%)	Peak height (mm)	Relative standard deviation (%)
1	35	28.1	0.99	72.2	3.13
2	43	33.6	0.81	164.0	2.18
3	57	44.0	0.97	229.6	3.55
4	59	46.2	0.88	157.4	1.04
5	63	50.5	1.11	31.7	2.23
6	73	60.8	1.03	153.1	3.19
7	74	62.1	1.06	80.9	1.97
8	92	92.0	1.08	91.8	1.64

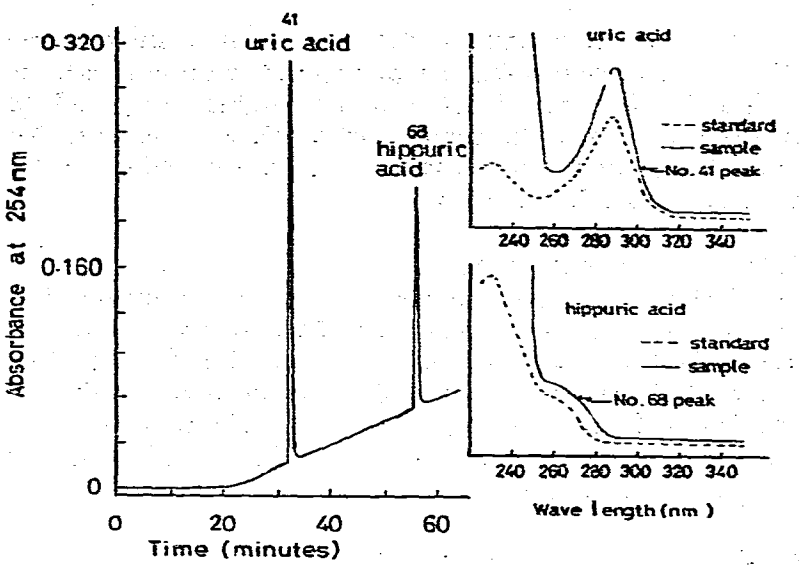

Fig. 3. Chromatogram of reference compounds and UV spectra of the peaks. Conditions are the same as in Fig. 1.

TABLE II

ASSIGNMENT OF THE MAIN PEAKS IN HUMAN URINE

Compound	Peak No.	Compound	Peak No.
Trigonelline	1*	Nicotinic acid	54
Creatine	3	4-Aminohippuric acid	55
Creatinine	3	2-Furoylglycine	57*
Pseudouridine	6*	5-Hydroxymethyl-2-furoic acid	59*
Pyridoxine	9	4-Hydroxy-3-methoxymandelic acid	65
Uracil	11	4-Hydroxy-3-methoxyphenylacetic acid	66
N'-Methyl-2-pyridone-5-carboxamide	17*	*p*-Aminobenzoic acid	67
Histidine	17	Hippuric acid	68
Theobromine	17	4-Hydroxy-3-methoxybenzoylglycine	70*
Nicotinamide	17	4-Hydroxybenzoylglycine	73*
7-Methylxanthine	17*	Quinaldic acid	73
Hypoxanthine	18	3-Hydroxybenzoylglycine	74*
Adenosine	19	*p*-Hydroxyphenylacetic acid	75
Phenylalanine	24	2,5-Furandicarboxylic acid	80*
Xanthine	25	Vanillic acid	76
3-Methylxanthine	26*	Kynurenic acid	85
Caffeine	29	*p*-Hydroxyphenylpyruvic acid	86
Tyrosine	29	Benzoic acid	88
Tryptophan	30	*p*-Hydroxybenzoic acid	88
Theophylline	30	3-Hydroxyanthranilic acid	89
1-Methylxanthine	32*	Indoleacetic acid	92
Urocanic acid	35	Indoleacrylic acid	101
Uric acid	41		
2-Amino-3-hydroxybenzoylglycine	43*		
Dimethyluric acid	48*		
5-Hydroxymethyl-2-furoylglycine	51*		

*Compounds assumed by comparison of the result established by Scott and Lee [21].

remarkably useful for the detection of the metabolites of the components of body fluids, such as purine, uronic acid, phenylalanine, tryptophan, benzoic acid, and their metabolites. The assignment of these normal urine components is important for application to the investigation or understanding of biological and pathological disorders.

Advantageous properties of the macroreticular anion-exchange resin

It is known that the macroreticular resins have a relatively higher cross-linkage, larger surface area, and larger porosity than the microreticular resins [16]. These macroreticular resins gave better adsorptivity, flow-resistance, mechanical stability, and chemical stability for ion-exchange chromatography.

With regard to the stability of Diaion CDR-10, it proved to be possible to analyze more than 200 urine samples over a 4-month period without any loss of resolution. Even when some decrease of resolution was noticed after applying a number of various types of samples, the column could be successfully recovered by washing with the dilute alkaline solution. Also, we have found that Diaion CDR-10 is mechanically stable when operated at high pressures and temperatures. We attribute this fact to a 35% cross-linkage which contributes to the mechanical stability of the resin and an easier chromatographic handling.

CONCLUSIONS

The purpose of this study was to use the macroreticular anion-exchange resin system for the separation of complex biochemical mixtures into their components and to examine the performance of the system [17]. The benefits of using macroreticular resin include a decrease in analysis time, an increase in resolution and a high degree of automation. Considerable work still remains to be done in the optimization of operating conditions, identification of the separated compounds, and also for application to clinical use. However, at this stage of development, the macroreticular anion-exchange resin system proved to be a more useful analytical tool than the previously developed systems of the microreticular or the pellicular anion-exchange resin columns, and is used already for routine analytical techniques in many medical research laboratories [18].

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