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SEPARATION OF URINARY ULTRAVIOLET-ABSORBING METABOLITES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY USING A COMMERCIALLY AVAILABLE ANALYTICAL UNIT

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SUMMARY

A high-pressure liquid chromatographic system for the separation of UV-absorbing and/or fluorescent urinary metabolites is presented. Thirty to forty UV-absorbing chromatographic peaks are obtained from a 200-μl urine sample using a 100 cm × 2 mm column filled with a total porous ion-exchanger. The reproducibility of the separation has been studied and eighteen important substances have been located.

INTRODUCTION

Human urine is reported to contain more than 700 molecular constituents [1]. About 150 of them absorb ultraviolet (UV) light [2]. These substances represent intermediates or end-products of metabolic pathways.

A physiological fluid as complex as urine can only be studied in detail by using a separation method with a high resolving power. A high-pressure liquid chromatographic (HPLC) system for the analysis of urine is presented, based on the fundamental work of Scott and co-workers [3—6].

Earlier HPLC systems are nowadays difficult to use because of the rapid evolution of HPLC technology and the advent of new commercial liquid chromatographs.

The analytical unit described has a very high resolving power, due to the use of a very fine, porous ion-exchanger, and gives reproducible results. It is versatile, easy to use and its components are commercially available. The detection system can be used for UV-absorbing metabolites, for reaction products absorbing in the visible region and for compounds that are naturally or artificially fluorescent.

EXPERIMENTAL

Materials

The high-pressure liquid chromatograph used is Model 8500 from Varian (Palo Alto, U.S.A.) equipped with a multilinear solvent programmer. The samples are injected via a liquid-chromatograph air-actuated sampling valve (sample loop of 200 μl, Varian) on a 100 cm × 2 mm I.D. 316 stainless-steel column. The columns are filled with Zerolit, a strongly basic, totally porous ion-exchanger. The column temperature is controlled by a Haake 22 water-bath with TP 32 programmator (Haake, Osterröde, G.F.R.).

The Variscan, a UV—vis continuous-wavelength detector with flow cells of 8 μl, and the dual-pen Techtron A-25 recorder, are associated Varian equipment. One pen of the recorder draws the absorption changes (line A in Fig. 2), the other the composition of the mobile phase expressed as the fraction of component B present in the eluent (line B in Fig. 2). The dual-wavelength detector is an ISCO UA5 absorbance monitor with a multiplexer-expander (Instrument Specialities Co., Lincoln, U.S.A.). The native fluorescence is continuously measured with a Fluorichrom (Varian).

The eluate is collected by a Redirac collector (LKB, Bromma, Sweden), which is cooled by placing it in a refrigerator (4°).

The fluorimeter is an Aminco-Bowman spectrophotofluorimeter (American Co., Silver Springs, U.S.A.).

Products

Zerolit (particle size, 5—7 μm) is a product of the Permutit (London, Great Britain) Aminex A-27 (particle size, 8—12 μm) can be obtained from Bio-Rad Laboratories (Richmond, U.S.A.). Acetic acid and ammonium acetate are pro analysi products from Merck (Darmstadt, G.F.R.). The standards are of the purest quality available from different sources.

Mobile phase

An acetic acid—ammonium acetate buffer (pH 4.4) varying from 0.015—6 *M* was used as the mobile phase.

METHODS

Fig. 1 is a schematic representation of the chromatographic system. Pumps A and B are filled, respectively, with 0.015 *M* and 6 *M* acetate buffer. Both solutions are previously de-gassed under vacuum.

The urine is collected, immediately centrifuged and 200 μl are promptly injected. The elution is programmed (Table I), the flow-rate is set at 8 ml/h and the chromatography is started. The column is maintained at 30° for the first 4 h; then the temperature is raised to 60° (1°/min) for the rest of the run. The UV absorption of the separated compounds is continuously measured and recorded at 254, 260 and 280 nm. In order to obtain a suitable absorption for the strongly absorbing components (creatinine, uric acid) as well as for the small peaks, an absorption range of 0—1 a.u.f.s. (on each detector) was found convenient for urine with a normal creatinine concentration (1000—2000 mg/24 h).

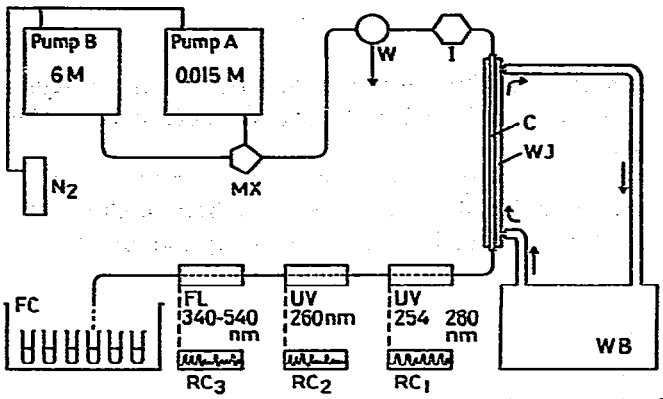

Fig.1. Schematic representation of the chromatographic system. C, column; FC, fraction collector; FL, fluorescence detector; I, injection system; MX, mixing chamber; RC, recorder; UV, UV absorbance detector; W, waste; WB, water-bath; WJ, water-jacket.

TABLE I

GRADIENT ELUTION PROGRAMME

Complete gradient elution takes 1140 min. The column can then be automatically regenerated by programming the 'reset'. In this position, the initial buffer composition (e.g. 0.015 *M*) will be pumped through the column. Regeneration can be followed by measuring the UV absorption (minimum regeneration time, 90 min).

Step*	Time (min)	Elution type	Buffer concentration (*M*)	
			beginning	end
1	120	Isocratic: (100% A + 0% B)	0.015	0.015
2	120	Linear gradient: (100% A + 0% B)— (96% A + 4% B)	0.015	0.3
3	600	Linear gradient: (96% A + 4% B)— (35% A + 65% B)	0.3	4
4,5,6	180	Isocratic: (35% A + 65% B)	4	4
7	35	Linear gradient: (35% A + 65% B)— (0% A + 100% B)	4	6
8	85	Isocratic: (0% A + 100% B)	6	6

*Step on solvent programmer

The fluorescence of the eluted compounds is continuously measured with the Fluorichrom (excitation wavelength 340 nm, emission wavelength 540 nm).

Finally, the eluate is collected into a refrigerated fraction collector in fractions of 1 ml to permit further study of the urinary metabolites by other techniques (fluorimetry, TLC, specific reactions).

RESULTS

The whole analysis takes about 20 h. The chromatograms from a fresh urine sample of a healthy adult are shown in Figs. 2 and 3. Thirty to forty UV-absorbing peaks are well separated.

The retention times (RT) of the compounds commonly present in urine have been determined and are given in Table II. Several other peaks were observed, which probably resulted from variations in the composition of the diet.

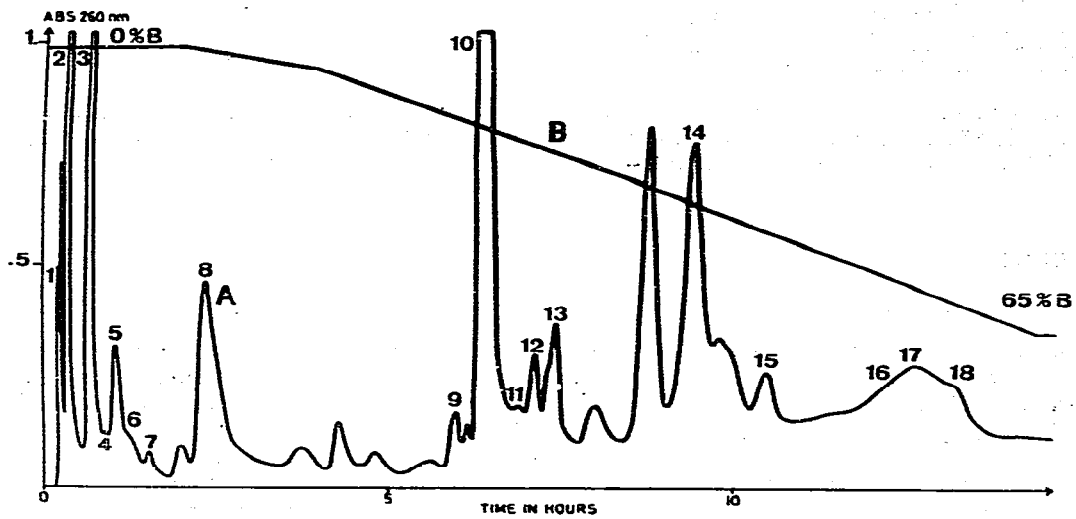

Fig.2. Typical chromatogram obtained using the Variscan detector. Conditions: flow-rate, 8 ml/h; eluant, ammonium acetate—acetic acid buffer (pH 4.4) varying from 0.015 to 6 *M* (buffer gradient as described in Table I); sensitivity, 1 a.u.f.s.; sample, 200 µl fresh urine of a healthy male subject (28 years old). Line A represents the absorbance at 260 nm, line B the buffer gradient expressed as the fraction of buffer B (6*M*) present in the mixture. Identified peaks (peak code number in parentheses): 1, N-methylnicotinamide (10); 2, creatinine (20); 3, pseudouridine (30); 4, tryptamine (31); 5, uracil (40); 6, serotonine (50); 7, urocanic acid (60); 8, nicotinamide (90); 9, hypoxanthine (130); 10, uric acid (160); 11, tryptophan (170); 12, 5-hydroxyindole acetic acid (180); 13, nicotinic acid (1801); 14, hippuric acid (240); 15, indican (250); 16, quinaldic acid (260); 17, vanillic acid (270); 18, kynurenic acid (2901).

Peak numbers are empirical, logical numbers. The RT of the urinary metabolites varies between 17 and 870 min. The peaks are well distributed over the whole chromatogram: 32% of the UV-absorbing metabolites are eluted during the first fifth of the run, 18% in the second fifth, 21% in the third, 16% in the fourth and 13% in the last fifth.

The chromatographic analysis is shown to be reproducible. The coefficients of variance lie between 8.7% (mean for the first six peaks, 7.6%) and 0.5% (mean for the last six peaks, 0.9%). As can be expected, the largest variations are noted in the first part of the chromatogram since separation in this region depends on non-ionic adsorption [8] which can be strongly affected by several factors.

The statistical study of the RTs of the separated compounds is compli-

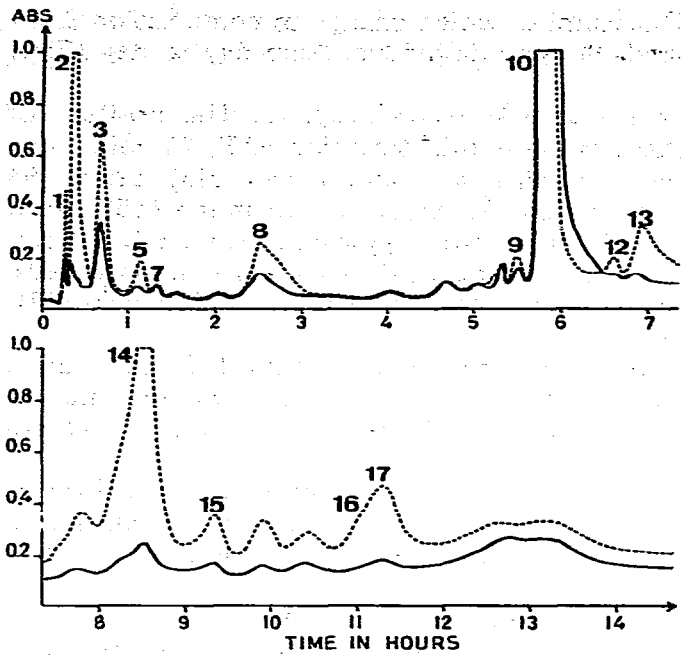

Fig.3. Chromatogram obtained using the ISCO UA5 absorbance monitor. - - - -, 254 nm; ——, 280 nm. For conditions and identified peaks, see legend of Fig. 2.

TABLE II

MEAN RETENTION TIMES OF THE SEPARATED PEAKS OF THE COMMON UV-ABSORBING COMPOUNDS IN URINE

RT, retention time. S.D., standard deviation. C.V., coefficient of variation (n = 10).

Peak number	Mean RT (min)	S.D.	C.V. (%)	Peak number	Mean RT (min)	S.D.	C.V. (%)
10	16.57	1.13	6.8	160	387.87	16.06	4.2
101	19.60	1.52	7.7	170	395.50	7.37	1.9
20	21.88	1.61	7.3	180	417.50	14.06	3.4
30	44.50	3.89	8.7	1801	431.33	12.13	2.8
40	63.60	4.81	7.5	190	453.28	12.71	2.8
50	73.50	5.43	7.4	200	476.37	13.07	2.7
60	87.83	3.49	4.0	210	483.40	4.98	1.0
70	96.00	4.90	5.1	220	506.00	10.20	2.0
701	114.33	2.94	2.6	230	545.17	2.86	0.5
702	127.00	1.00	0.8	231	557.80	3.90	0.7
80	142.40	8.16	5.7	240	578.37	9.01	1.6
90	153.75	9.60	6.2	250	605.00	9.43	1.6
901	188.00	5.65	3.0	260	633.57	2.94	0.5
100	206.17	8.68	4.2	2601	666.57	7.55	1.1
110	266.80	7.43	2.8	270	709.33	7.45	1.0
120	286.57	10.87	3.8	2701	733.00	8.66	1.2
130	310.57	10.11	3.3	280	770.00	6.00	0.8
140	329.66	17.08	5.2	2901	844.25	4.19	0.5
150	353.60	11.70	3.3	2902	870.50	6.36	0.7

cated by the variability of urine samples, which change in composition from person to person and for one and the same individual from day to day (Figs. 2, 4 and 5 and Table III).

Identification of the urinary metabolites is in progress. The position of 18 of them has been determined: N-methylnicotinamide (RT, 17 min), creatinine (22 min), pseudouridine (45 min), tryptamine (58 min), uracil (64 min), serotonin (72 min), urocanic acid (89 min), nicotinamide (158 min), hypoxanthine (308 min), uric acid (380 min), tryptophan (395 min), 5-hydroxyindole acetic acid (418 min), hippuric acid (575 min), indican (610 min), quinaldic acid (634 min), indolacetamide (664 min), vanillic acid (710 min) and kynurenic acid (844 min).

This identification is based on the absorption ratio (280/254 nm), native fluorescence, co-chromatography, retention time of pure products and, if possible, fluorimetric spectra, TLC and specific reactions [10]. The identified peaks are indicated in Fig. 2.

Identification is made difficult by the fact that only small quantities of metabolites are involved. Concentration techniques, (such as lyophilisation and evaporation under vacuum), have been shown to degrade some labile compounds [10].

Fig. 6 shows the fluorescent compounds present in urine.

The buffer gradient designed for the Zerolit column can also be used with an Aminex A-27 column (100 cm × 2 mm) as illustrated in Fig. 7, although a slight modification of the flow-rate programme is necessary (see legend to Fig. 7).

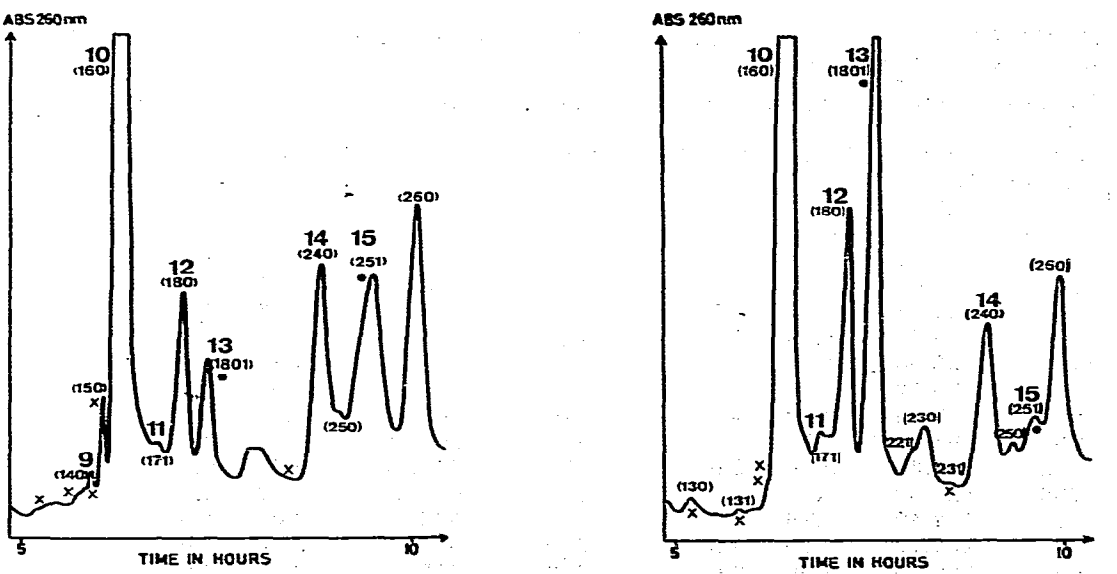

Fig.4. Detail of a chromatogram of a 200-μl urine sample from the same person as in Fig. 2. Conditions and identified peaks as described in Fig. 2. Note the quantitative (*) and qualitative (×) differences with Fig. 5. In parentheses, peak code number.

Fig.5. Detail of a chromatogram from a second male subject (45 years old). Conditions and identified peaks as described in Fig. 2. Note quantitative (*) and qualitative (×) differences with Fig. 4. Peak code numbers in parentheses.

TABLE III

COMPARISON OF PEAK AREA OF URINE SAMPLES

U_{10}, U_{20}, U_{30}, U_{40}: urine samples from 4 male subjects (age 28—50 years); U_{40}, U_{41}, U_{42}, and U_{43}: urine samples from the same person (28 years), taken on different days. Peak area was calculated using the formula: peak area = height × width at half height (No molar response factors were taken into account since some peaks are still unidentified). n.d. = not detectable; n.m. = not measurable because out of scale.

Peak code number	Peak area (mm^2)							
	Samples from different persons				Samples from one person			
	U_{10}	U_{20}	U_{30}	U_{40}	U_{40}	U_{41}	U_{42}	U_{43}
10	216	227	151	165	165	186	132	165
101	484	79	182	177	177	n.d.	176	130
40	55	n.d.	n.d.	103	103	trace	21	22
90	656	420	617	44	44	900	806	28
1701	184	150	74	n.d.	n.d.	trace	47	trace
180	234	180	632	465	465	598	198	276
1801	461	680	n.m.	21	21	601	368	trace
240	1160	539	61	1680	1680	1508	1282	1371
250	57	72	63	125	125	trace	60	99
260	469	294	691	1000	1000	531	n.d.	642

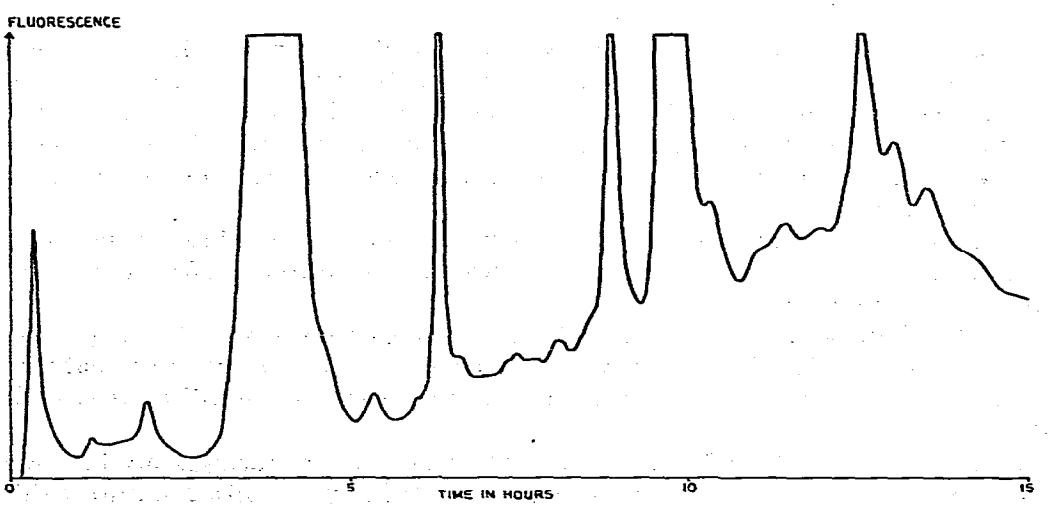

Fig.6. Chromatogram showing the fluorescent compounds in urine. Excitation wavelength 340 nm, emission wavelength 540 nm. Attenuation, 20; gain, 1. Identification of these peaks is in progress.

DISCUSSION

The whole of the chromatographic system used is commercially available (in contrast to the apparatus used by the Oak Ridge group, which was a self-constructed high-pressure liquid chromatograph).

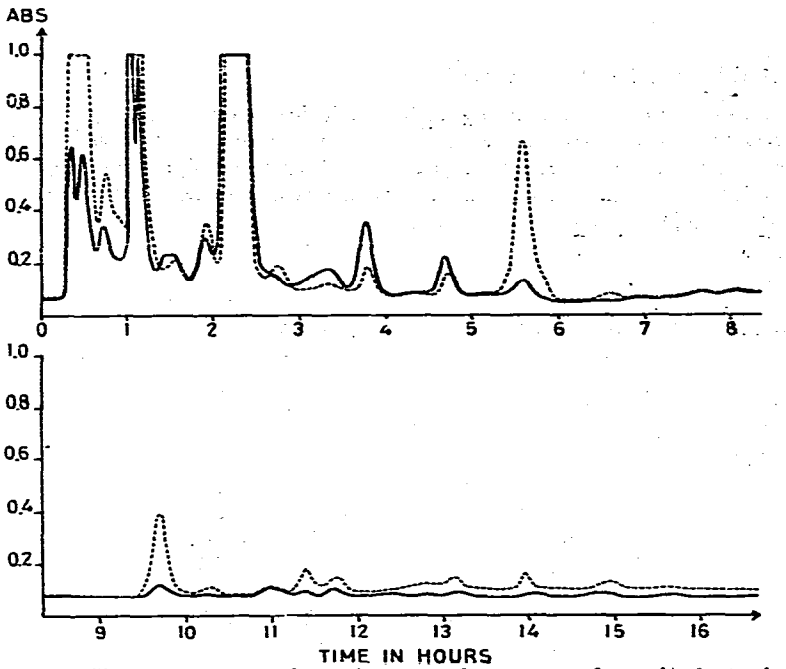

Fig.7. Chromatogram of a urine sample separated on Aminex A-27. Flow rate programme: 0—2 h, 4 ml/h; 2—19 h, 8 ml/h. Gradient programme: see Table I. - - - -, 254 nm. ——, 280 nm.

The buffer system is that chosen by Scott et al. [2], and it has also been used by Burtis [9]. As the components of the buffer (acetic acid and ammonium acetate) have the advantage of being volatile, they can be evaporated during concentration procedures (lyophilisation) in order to avoid eventual interference during TLC and other procedures where the presence of salts is not desirable.

The buffer gradient and the flow-rate have been determined experimentally [10] and result from a compromise between separation performance and speed for the resin used.

Recently, Zerolit and Aminex A-27 have been compared [7]. The plate height for the purified Zerolit (which has been used as the packing material of the column) has been shown to be about seven times smaller than that with Aminex A-27.

Raising the temperature from 30° to 60° after 4 h chromatography has two advantages: a 50% decrease in the RT of strongly adsorbed components [7], and a lowering of the viscosity of the concentrated buffer, which results in a fall in the pressure drop.

Liquid chromatography is shown to be a useful technique for the separation of urinary constituents. The method is reproducible and the detectors presently available allow detection at the nanogram level, which formerly could only be attained by gas chromatography. However, this separation technique requires the transformation of non-volatile compounds into volatile derivatives. With HPLC, a urine sample can be analysed without derivatization, which eliminates the risk of degradation prior to chromatography.

With regard to the stability of the column packing material, daily use for 6—7 months is possible. Tailing and rise in pressure (normal starting pressure is about 2000 p.s.i., end pressure about 4000 p.s.i.) are signs that the column needs to be changed. Intercolumn differences are within the normal chromatogram-to-chromatogram variation.

The HPLC system described here has been used in our laboratory for more than a year without presenting the great practical difficulties often mentioned which until now have retarded the introduction of this new technique into pharmacological and clinical laboratories.

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