CHROM 4890

THE SEPARATION OF THE ULTRAVIOLET-ABSORBING CONSTITUENTS OF URINE BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-resolution liquid chromatograph has been used to separate more than ninety ultraviolet-absorbing constituents from human urine. A 200 μl urine sample was chromatographed on a 0.24 × 100 cm column packed with 12–15 μ anion exchange resin. Over ninety ultraviolet-absorbing constituents were separated in less than 20 h by eluting with a linear acetate gradient at a flow rate of 8 ml/h and a pressure of 1000–1600 p.s.i. The column temperature was maintained at 21° for the first 4 h and then increased to 60° for the final 16 h of the run. The column effluent was monitored with an ultraviolet photometer that permitted absorbance to be recorded linearly at scales ranging from 0.02 to 0.64 absorbance unit full scale. A relative standard deviation of 1–4% was observed between the elution times of similar peaks from three identical chromatographic runs. The advantages of pre-fractionating urine by DEAE chromatography before anion-exchange chromatography was demonstrated.

INTRODUCTION

Body fluids such as urine contain many compounds that have been shown to have a correlation with various pathological states[1]. Recently, PAULING[2] has indicated that the concentration of even normal constituents is important in the maintenance of proper mental health. It is obvious that the qualitative and quantitative knowledge of the normal or pathologic constituents in body fluids would be beneficial in the evaluation of body function. Unfortunately, these constituents are contained in a complex biological mixture and often must be separated before qualitative and quantitative analysis may be performed. In several instances a specific colorimetric or enzymatic method is used for the analysis of a single constituent, but these methods invariably suffer from interference from other constituents contained in the body fluid[3].

SCOTT and co-workers at the Oak Ridge National Laboratory have developed a system, designated the "UV Analyzer", which separates the UV-absorbing constituents of body fluids by high-resolution anion-exchange chromatography[4–8].

With this system they have demonstrated the separation of more than one hundred UV-absorbing constituents from human urine in less than 40 h. Several of these separated constituents have been qualitatively identified[6,9–10] but this has proved difficult due to the small sample quantities involved and their resultant dilution by the eluting solvent.

A different approach is that of using the chromatogram, obtained with the "UV Analyzer", as a profile or fingerprint in evaluating body function. Reference profiles of "normal" subjects are then obtained and compared with profiles from patients having various metabolic or pathologic abnormalities. Using this approach, emphasis must be placed on obtaining accurate and precise reference profiles. We report here, using the chromatographic procedure developed by SCOTT *et al.*[4], that reproducible, urinary profiles may be accurately and precisely obtained with a commercially available liquid chromatograph.

EXPERIMENTAL

Materials

Chemicals. The 0.015 *M*, pH 4.40 and 6.0 *M*, pH 4.40 sodium acetate buffers were prepared from analytical grade sodium hydroxide and acetic acid. The reference compounds, known to be in urine, were purchased from Calbiochem, Los Angeles, Calif., U.S.A.

Resins. The anion-exchange resin Aminex BRX having a nominal 8% divinylbenzene cross-linkage and a particle size distribution of 12–15 μ was a gift from Bio-Rad Laboratories, Richmond, Calif., U.S.A. The resin, as received from the manufacturer, was swollen for 24 h in 0.015 *M* sodium acetate, pH 4.40 before column packing. The DEAE-Sephadex was purchased from Pharmacia, Piscataway, N.J., U.S.A.

Column preparation. A 0.24 × 100 cm stainless-steel column was dynamically packed with the Aminex BRX anion resin as described by SCOTT AND LEE[11]. An extension (0.54 × 25 cm) was added to the column and both were filled with a 50:50 slurry of the resin and 0.015 *M* sodium acetate, pH 4.40. The column was packed at 1000 p.s.i. using a Milton Roy Minipump and equilibrated for several hours with the acetate buffer. The extension was then removed and the column placed in the chromatographic system.

Chromatographic system. The system used was the Varian Aerograph Model LCS-1010 Liquid Chromatograph, a schematic of which is shown in Fig. 1. The system is capable of gradient elution at pressures up to 3000 p.s.i. The gradient is formed by pumping (Pump 1) a solution of concentrated eluent from Buffer Reservoir 1 into the gradient chamber previously filled with a predetermined volume of dilute eluent from Buffer Reservoir 2. The resultant gradient is pumped through the column by means of a Milton Roy Milroyal D (Pump 2), which is capable of leakfree operations up to 3000 p.s.i. A linear gradient is produced by maintaining the flow rate through the column at twice the rate of flow of the concentrated eluent into the gradient chamber[12]. The initial volume of dilute eluent in the gradient chamber determines the slope of the linear gradient. Both convex and concave gradients in solvent concentration with time can be generated by adjusting the relative flow rates into and out of the gradient mixing chamber. The system has a timing delay which

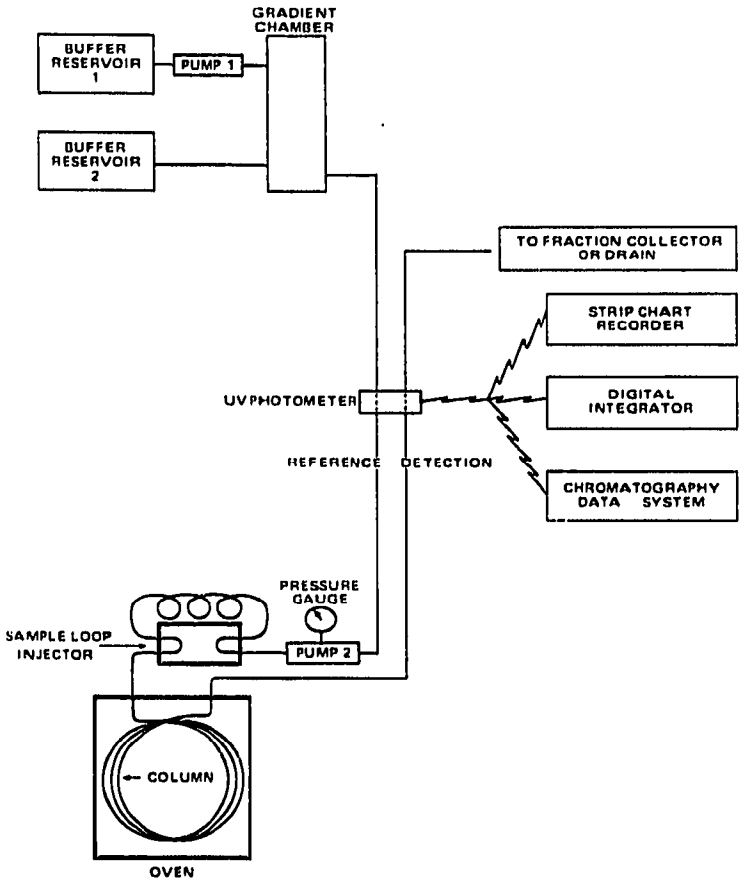

Fig. 1. Schematic of the Varian Aerograph Model LCS-1010 Urine Analyzer.

allows a timed elution with the dilute buffer (*i.e.*, up to 30 min) before the start of the gradient elution. The sample is introduced into the column through a sample loop injector at the top of the instrument. The column is housed in an air oven which is capable of maintaining the column temperature to within $\pm 1°$ of the selected setting. The column effluent is monitored by a sensitive UV photometer, operating at 254 nm, which is equipped with a cylindrical flow cell having a 1 mm diameter and 10 mm path length (*i.e.*, 8 μl cell volume). The detector output is linear in absorbance units and is linear with respect to solute concentration in accordance with Beer's Law. Full scale absorbance ranges from 0.02 up to 0.64 are provided in binary steps. In addition, a nonlinear high absorbance range is provided for qualitative monitoring of highly absorbing samples. The photometer output is displayed on a 10 mV strip chart recorder or fed into a digital integrator or a chromatography data handling system for data acquisition and processing. To minimize baseline drift, due to the UV absorbance of the concentrated acetate buffer, the gradient stream flows through the reference cell in the UV photometer before it enters the 3000 p.s.i. pump.

The column effluent, after passing through the sample cell of the detector, is routed either to a drain or to a fraction collector.

Sample preparation. Urine samples were collected from healthy male subjects and were refrigerated between the time of collection and the time of analysis. A

urine sample, that had been fractionated into an acidic fraction and into a combined basic and neutral fraction by DEAE chromatography, was provided by Dr. LINUS PAULING and Dr. ART ROBINSON of Stanford University.

Anion-exchange chromatography. To introduce a urine sample onto the column, the eluent flow was stopped, the pressure reduced, and the 200 μl sample loop filled with urine. The urine sample was then eluted with a linear acetate gradient at a flow rate of 8 ml/h. The column pressure required to maintain this flow rate varied from 1000 to 1600 p.s.i. depending on the column temperature and the viscosity of the acetate gradient. The linear acetate gradient was formed by placing 90 ml of 0.015 *M* sodium acetate, pH 4.40 in the gradient chamber and pumping into it 6.0 *M* sodium acetate, pH 4.40 at a flow rate of 4 ml/h. As mentioned earlier, the resulting gradient is delivered to the column at a flow rate of 8 ml/h. The optimal column temperature, as determined by SCOTT *et al.*[4] was maintained at 21° for the first 4 h and increased to 60° for the final 16 h of a chromatographic run. Between runs the column was equilibrated with the 0.015 *M* sodium acetate for 4 h. Thus, allowing 20 h for the chromatography and 4 h for equilibration, 1 sample may be chromatographed per 24-h day.

DEAE chromatography. A urine sample was fractionated into an acidic fraction and into a combined basic and neutral fraction by modifying the chromatographic procedure of HORNING[13]. DEAE-Sephadex (diethylaminoethyl-Sephadex), a weakly basic anion-exchange support, was suspended in distilled water, washed with 0.5 *N* sodium hydroxide and rewashed with distilled water to pH 7.0. The water was decanted, the resin resuspended in 0.01 *M* pyridine acetate and the pH adjusted to 5.0 with glacial acetic acid. A 2 × 10 cm glass column was then filled with a slurry of the resin and the resultant column washed with 50 ml of distilled water. A 10 ml urine sample was allowed to percolate into the column and the basic and neutral urinary fraction eluted with 100 ml of distilled water. The acidic fraction was then eluted with 150 ml of 1.5 *M* pyridine acetate. The 100 ml water fraction containing the basic and neutral constituents and the 150 ml pyridine acetate fraction containing the acidic constituents were both lyophilized to dryness. The dry samples were then reconstitued to 10 ml with distilled water and 200 μl aliquots of each and a 200 μl aliquot of the unfractionated urine chromatographed by anion-exchange chromatography.

RESULTS

A typical urine chromatogram of a 100 kg male subject is seen in Fig. 2. The column resolved 99 UV-absorbing peaks from this particular urine. By increasing the column length from 100 to 200 cm we have been able to increase the number of resolved peaks to more than 110, but at the expense of increasing the analysis time to 45 h. Therefore, in the subsequent runs we have utilized the 100 cm column and an analysis time of 20 h and have found these conditions to be the best compromise between resolution and length of analysis time. In addition, the profile of the acetate gradient employed in this (Fig. 2) and subsequent separations is indicated.

Although identification of every individual peak is difficult, a few peaks may be identified by comparing elution times with reference compounds. For example, in Fig. 2, the identity of the uric and hippuric acid peaks eluting at 5.3 and 13.4 h,

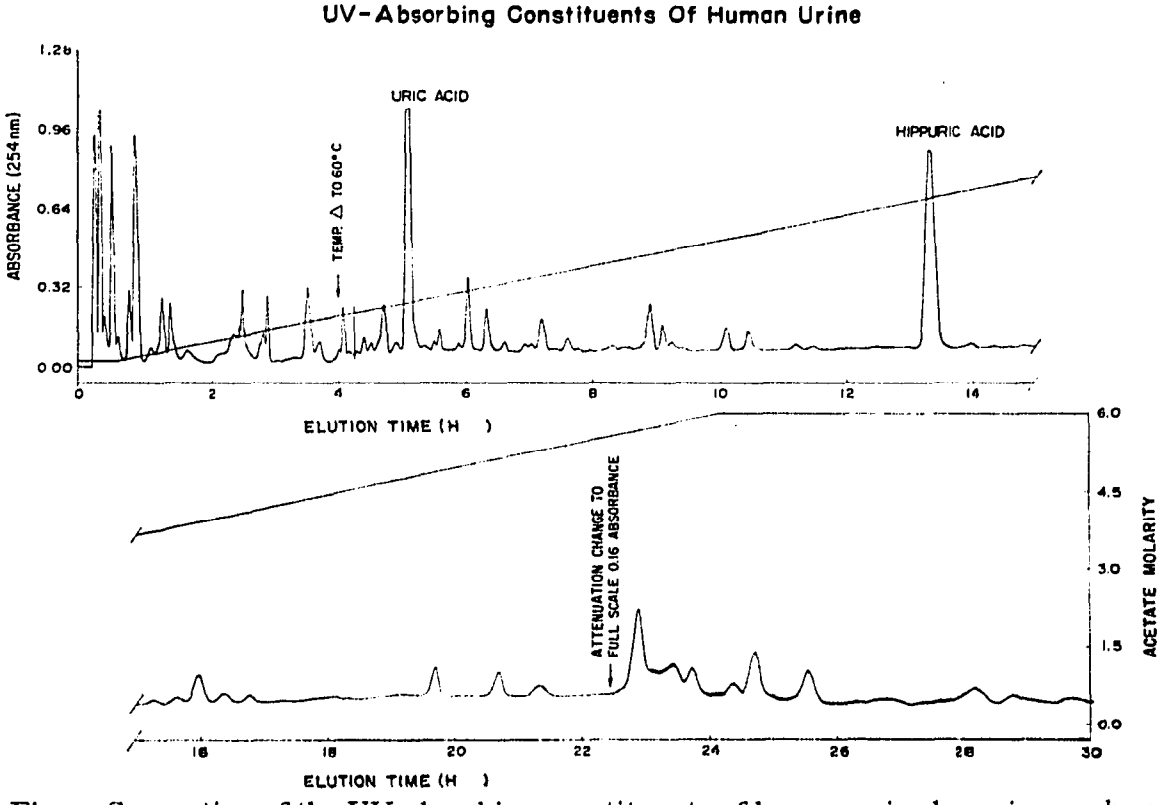

Fig. 2. Separation of the UV-absorbing constituents of human urine by anion-exchange chromatography. Conditions: columns, 0.24 × 100 cm; resin, Aminex BRX (12–15 μ) strongly basic anion exchanger; eluent, linear sodium acetate gradient varying in concentration from 0.015 M, pH 4.4 to 6.0 M, pH 4.4; flow rate, 8 ml/h; pressure, 1000–1600 p.s.i.; column temperature, ambient to 4 h, 60° for remainder of run; sample, 200 μl human urine.

respectively, was determined by comparing their elution times with those of reference uric and hippuric acid. Since this type of identification, and problems associated with it, have been discussed previously[6,9] the following chromatograms will be discussed on the basis of profiles instead of individual peak identities.

TABLE I

PRECISION OF THE ANION EXCHANGE CHROMATOGRAPHIC METHOD USED TO SEPARATE THE UV-ABSORBING CONSTITUENTS OF URINE

Numbers represent the mean ± the relative standard deviation (column 3) for 3 determinations (Fig. 3).

Peak number	*Elution time (min)*	*Relative standard deviation (%)*
1	30.9	3.9
2	53.8	4.2
3	85.8	2.5
4	205.6	2.1
5 (Uric acid)	285.8	1.7
6	486.6	2.6
7 (Hippuric acid)	701.5	1.7
8	822.6	1.9
9	968.7	1.1
0	1003.6	2.5

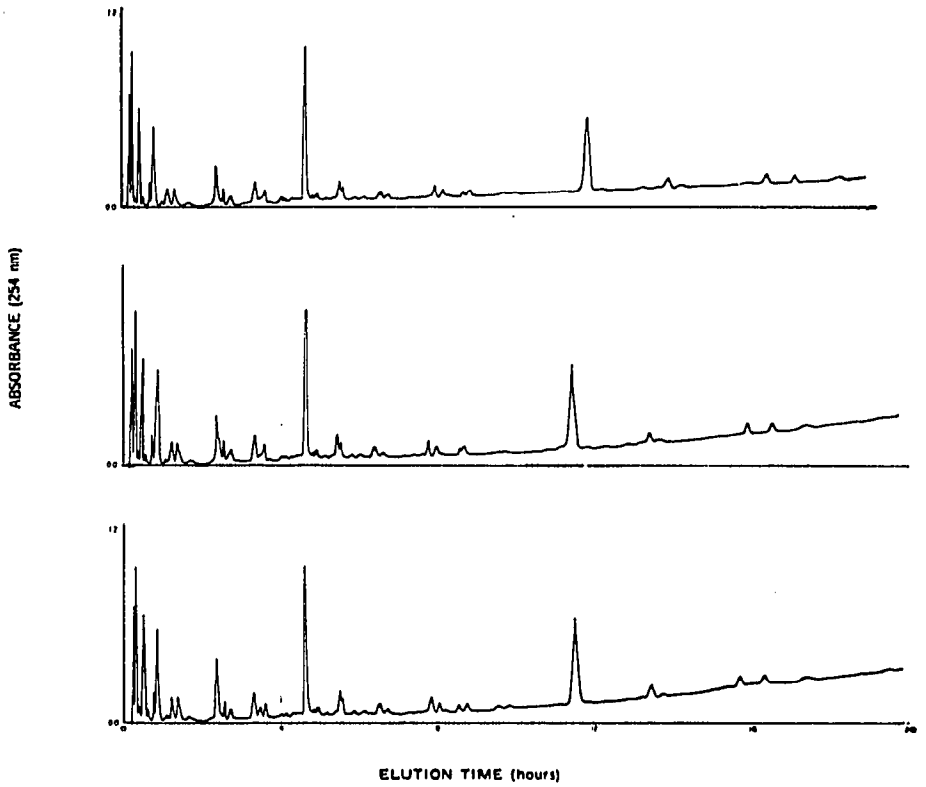

Fig. 3. Reproducibility of the separation of the UV-absorbing constituents of human urine by anion-exchange chromatography. Chromatograms resulted from three identical chromatographic analyses using the same conditions listed under Fig. 2. The 200 μl sample was the same urine in all three cases.

To demonstrate the reproducibility of the chromatographic procedure, three identical chromatographic analyses were performed using aliquots of the same urine sample. The three resultant chromatograms are seen in Fig. 3. The elution times of 10 reference peaks were determined and found to have a relative standard deviation of $\pm$1–4% (Table I). As expected, the greatest deviation between elution times was found in the early region of the chromatograms since separation here is dependent on non-ionic adsorption[9], which is strongly affected by many factors. Compounds that are more strongly retained by the resin (*i.e.*, compounds that elute in the 2–20 h region of the chromatogram) are not markedly influenced by non-ionic factors and therefore the elution times, of similar peaks in this region, have relative standard deviations of only $\pm$1–3%.

To determine the precision of the method on a long term basis, the elution times for uric and hippuric acid from 15 chromatographic runs were obtained and found to have a relative standard deviation of $\pm$2.8 and 3.0%, respectively. Thus, with a precision of $\pm$1–4%, it is obvious that the chromatographic procedure is reproducible, which is mandatory when one attempts to detect and correlate differences in urinary profiles.

It has been previously reported[6,9] that peaks representing a class of compounds will elute at certain times from an anion-exchange column. For example, basic and neutral compounds elute in the first few hours while the acidic compounds elute much later in a chromatographic run. To confirm this, urine was prefractionated

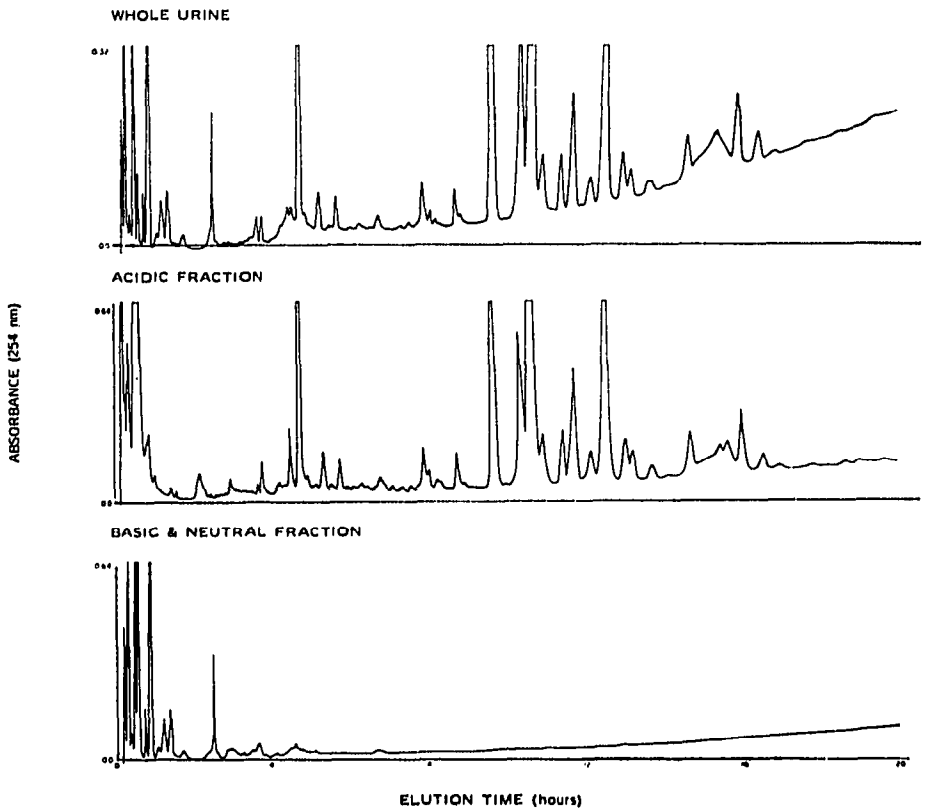

Fig. 4. The separation of the UV-absorbing constituents of DEAE-fractionated urine. The top chromatogram represents the profile of a whole urine sample. The same urine was fractionated into an acidic fraction and into a basic and neutral fraction by DEAE chromatography and then chromatographed by anion-exchange chromatography. The middle and bottom chromatograms represent the acidic fraction and the basic and neutral fraction, respectively. Chromatographic conditions are the same as listed in Fig. 2.

into an acidic fraction and into a combined basic and neutral fraction by DEAE chromatography. Aliquots of these fractions were then chromatographed by anion-exchange chromatography which resulted in the following chromatograms (Fig. 4). The first chromatogram was obtained from the chromatography of an aliquot of the whole urine. The second and third chromatogram represent the acidic and the combined basic and neutral fractions, respectively.

It is evident that the acidic, basic and neutral components of urine elute at discrete intervals from the anion-exchange column. For example, the 4–20 h region of the whole urine chromatogram correlates with the 4–20 h region of the acidic fraction chromatogram. This indicates that the acidic urinary components are eluting in this time interval, an observation which is consistent with previous studies[6,9]. On the other hand, the chromatogram of the basic and neutral fraction correlates with the 0–3 h region of the whole urine chromatogram indicating that the basic and neutral urinary constituents are eluting in this time interval. In addition, there appears to be a region of overlap at 2–4 h where either acidic, basic or neutral components may elute.

In the chromatogram of the acidic fraction, several compounds elute at 1–2 h from the anion-exchange column which do not appear in the chromatogram obtained

from the whole urine. It is probable that these peaks are due to the elution of the neutral or aglycon moieties of conjugated compounds (*i.e.*, glucuronides, sulfates, phosphates, etc.) that arise from the hydrolysis of the conjugates by the chromatographic conditions of the DEAE chromatography. The peaks representing the conjugated compounds could be ascertained by first hydrolyzing the urine, either enzymatically or chemically, prior to the anion-exchange chromatography. Thus, by comparing the resulting anion-exchange chromatogram with that obtained from unhydrolyzed urine, the peaks that correspond to conjugated compounds could be clearly determined. Previously, this approach has been utilized to isolate the aglycon moiety of the conjugated metabolites of phenacetin[9]. In addition, being able to correlate definite peaks with conjugated compounds would be useful since the degree of conjugation is often used as a basis for liver function tests[14,15].

DISCUSSION

The results presented here confirm the data of SCOTT and co-workers who have previously demonstrated the applications and advantages of separating the UV-absorbing constituents of urine by high pressure anion-exchange chromatography. In earlier reports SCOTT and his group have used this technique to evaluate urine from leukemic and schizophrenic patients[4], the diurnal pattern of a normal male human[5], a 2-year old girl who had a neuroblastoma[10] and other pathological states such as hereditary nephritis, Lesch-Nyhan syndrome, gout, alkaptonuria and maple syrup urine disease[16]. In addition, VAVICH AND HOWELL[17] and KELLEY AND WYNGAARDEN[18] have used an Oak Ridge Analyzer in the evaluation of urine for its UV-absorbing compounds from normal newborn and young children and patients with gout, respectively.

Thus, liquid chromatography has been demonstrated to be a particularly useful technique for the separation of complex biological mixtures into their individual components. For example, it allows one to separate a highly complex mixture such as urine, since in liquid chromatography one can easily vary many chromatographic parameters. In addition, we have found the technique to be reproducible to within 1–4%. Assuming a molar absorptivity of 10,000, ng quantities of UV-absorbing urinary constituents can be detected and quantitatively determined. Previously, one was only able to obtain this degree of sensitivity with gas chromatography. However, the gas chromatographic analysis of urinary constituents usually requires first a derivatization procedure consisting of several reaction steps. Using liquid chromatography, a sample can be analyzed directly which eliminates the concern for quantitative derivatization or chemical degradation arising from the derivatization procedure.

To obtain the high resolution necessary in the separations presented here the use of a column filled with an efficient ion-exchange resin of small particle size is required[4]. This small resin, however, requires operation at high pressures to obtain usable flow rates. Thus, a system to perform these separations must be capable of (1) continuous operation at high pressures, (2) must be compatible with high-efficiency columns, and (3) must have a sensitive column monitor to quantitatively detect the minute quantities of solutes that are separated by these high resolution columns. We have found the LCS-1010 Urine Analyzer to meet all of the above

requirements. The pumping system of the LCS-1010 is capable of reliable operation at pressures up to 3000 p.s.i., and we have observed only a 1–4% deviation in the elution times of similar peaks, which indicates a stable and reproducible flow rate.

The UV photometer has been discussed earlier and it was shown to be capable of detecting ng quantities of UV-absorbing compounds[12,19]. An additional advantage, apart from its high sensitivity, is the fact that its output is linear in absorbance, thus linear in concentration. This allows for data acquisition by a digital integrator or computer data system in addition to the readout obtained from a strip chart recorder. In the future, this digital collection of data will prove useful when data from two urinary profiles are compared either manually or, preferably, with a computer.

In regards to the chemical stability of the anion-exchange resin used in this study, we have analyzed, on a single column, more than 60 urine samples over a 9-month period without loss of resolution. This agrees well with the results of SCOTT *et al.*[4] who have analyzed over 100 urine samples on a single column without encountering any evidence of chemical degradation of their resin.

In addition to the chemical stability of the resin, we have found the BRX anion-exchange resin to be mechanically stable when operated at high pressures. We attribute this to the fact that we have used resin of 8% cross-linkage* which contributes to the mechanical stability of the resin. Also, the BRX resin was a narrow cut of small particle size which, when "dynamically packed", has been shown by SCOTT AND LEE[11] to yield a uniformly packed column bed that is operable at high pressures.

In conclusion, we have reconfirmed, using a commercially available liquid chromatograph, that high-pressure anion-exchange chromatography is a useful technique for the separation of the UV-absorbing constituents of human urine. The resultant chromatograms or profiles are an indication of the metabolic state of a subject and should prove useful in the diagnosis of several abnormal metabolic or pathological conditions. However, to use such profiles for diagnostic purposes, dietary effects must be considered as YOUNG[20] has observed different urinary profiles from the same individual when different diets have been ingested. Consequently, in conjunction with Dr. L. PAULING and Dr. A. ROBINSON of Stanford University, we are presently analyzing urine samples obtained from subjects on a chemically defined diet. This should allow us to ascertain or minimize the effect of diet on urine composition which is necessary if urinary profiles are to be utilized for diagnostic purposes.

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* % cross-linking indicates the % of divinylbenzene that is incorporated into the polymer bead prior to attaching ionic groups.

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