High-Resolution Chromatography of Complex Organic Mixtures

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The high-resolution uv analyzer has been used to investigate the effect of diet on urine profile. In addition, new applications in the food and beverage industry appear promising. More than 100 compounds, acidic to basic, can be separated. The clinical analyst may be interested in scanning a wide variety of compounds or in rapid separation of only a few compounds. For the latter purpose, the effect of changing the gradient elution program has been investigated, and conditions have been defined for rapid analysis of specific regions of the chromatogram. Additionally, the chromatogram may be simplified by fractionation—for example, by solvent extraction—before high-resolution analysis. The sensitivity of the instrument is often limited by baseline drift caused by absorbance of the buffer. Various methods for compensating for this drift have been investigated. Reproducibility of retention time, response, and computerized data processing are discussed.

Additional Keyphases  
- fractionation of urine, vanilla, wine
- dietary effects
- speeded analyses
- prechromatography fractionation of urine
- baseline drift
- and compensation
- gradient elution

Scott and co-workers at the Oak Ridge National Laboratory have developed an instrument that separates the uv-absorbing constituents of body fluids by high-resolution liquid chromatography (1–6). By using columns filled with efficient anion exchange resin of small particle size and operating at high pressures, they have separated more than 100 uv-absorbing constituents from human urine in less than 40 h.

This instrument has been used to examine urine from leukemic and schizophrenic patients, from a normal male human [to learn more about diurnal variation (2)], from a 2-year old girl who had a neuroblastoma (7), and from patients with other pathologic conditions such as hereditary nephritis, Lesch–Nyhan syndrome, gout, alkaptonuria, and maple-syrup urine disease (6). Other investigators have used this instrument to examine the urine from newborns and young children (8), patients with gout (9), and to survey the effect of diet on the chromatographic profile (10).

These studies have demonstrated that liquid chromatography is a useful technique for evaluating body function. The chromatograms are metabolic profiles of the individual subjects, and thus indicate the state of their metabolic processes at the molecular level.

Because of the obvious significance of this work, we decided to investigate the possibility of modifying an existing instrument, the LCS-1000, to perform the analysis. The various instrumental parameters, such as gradient-elution program and detector-cell plumbing, were of special interest. Also, the reproducibility of columns and instruments was investigated.

Materials and Methods

Materials

Chemicals. Acetate buffer, 0.015M (pH 4.40) and 6.0M (pH 4.40) (25°C), were prepared from AR grade sodium hydroxide and acetic acid by adding NaOH to the appropriate concentration of acetic acid until the desired pH was obtained.

The reference compounds, known to be in urine, were purchased from Calbiochem, Los Angeles, Calif. 90063.

Column resin. The strongly basic anion-exchange resin Aminex-27 (control no. 9242) having a nominal 8% divinylbenzene cross-linkage and a particle diameter distribution of 12–15 μm, was obtained from Bio-Rad Laboratories, Richmond,
The cation-exchange resin, used for the pre-fractionation of urine, was AG 50W-X8 (Bio-Rad) 200–400 mesh, hydrogen form.

Solvents. Organic solvents, ethyl acetate and, chloroform, were “spectrograde” (MC & B Mfg. Chemists, Norwood, Ohio 45212).

Liquid Chromatograph

The instrument used for urine analysis is a Model LSC-1010 (Varian Aerograph, Walnut Creek, Calif. 94598), a high-pressure liquid chromatograph especially modified for injection of 200-μl samples (Figure 1). It consists of a gradient generator, high-pressure pump, high-resolution anion-exchange column, uv detector, and associated data-recording and -processing devices. Two precision metering pumps are used to form the solvent gradient. The gradient chamber is first filled with a volume of dilute eluants from buffer reservoir 2. With pump 1, the concentrated buffer in reservoir 1 is pumped into the gradient chamber, where the solutions are mixed thoroughly. The resulting solution is then forced through the column by pump 2, which can produce pressures as great as 21 × 10³ N/m² (3000 psi). A linear gradient is produced when the flow rate through the column is twice that of the concentrated eluant into the gradient chamber. The slope of the linear gradient is determined by the initial volume of dilute eluant in the gradient chamber, and by the flow rates of the two pumps. Concave or convex gradients may also be formed. The system has a timer that may be used to delay the beginning of the gradient elution.

Sample injector. Samples are introduced onto the column by use of a sample-loop injector, stopping the flow of buffer. The volume of sample can be varied from 20 to more than 200 μl, depending on the dimensions of the removable sample loop. The capacity of our sample loop was 200 μl.

Column. A 0.24 × 100 cm stainless-steel column was dynamically packed with the Aminex A-27 anion-exchange resin as described by Scott and Lee (11). A precolumn (0.54 × 25 cm) was added to the column and both were filled with a slurry of the resin in an equal volume of 0.015M buffer. The column was packed at 6.89 × 10⁴ N/m² (1000 psi) by use of a “Minipump” (Milton Roy Co., St. Petersburg, Fla. 33733). After equilibrating the columns for several hours with the acetate buffer, we removed the precolumn and placed the column in the chromatograph. The resin bed is supported by a 1/8 in. union containing a 0.5-μm stainless-steel frit (Crawford Fitting Co., Solon, Ohio 44139). The 100-cm column is housed in an air oven capable of maintaining the column temperature to within ±1°C of the selected setting.

UV photometer. The uv absorbance of the column effluent is monitored by a sensitive uv photometer that has a low dead-volume. The photometer can measure the absorbance at 254 nm. In the dual-column mode of operation, the detector monitors the difference in absorbance between the analytical and reference column effluents. Normally, the reference cell was filled with air. The detector output is linear in absorbance units and therefore directly proportional to concentration for solutes that obey Beer’s law.

Two dual-flow cells were used. The more sensitive version has cells with an internal diameter of 1 mm and a path length of 10 mm, which gives a dead volume of 8 μl. A cell with a shorter path length was used for higher concentrations. The construction was similar to the 10-mm cell except that the path length was 4 mm. The detector can be operated over a linear absorbance scale ranging from 0.02 to 0.64 absorbance units (full scale). Higher absorbance values are recorded on a non-linear scale.

Baseline Compensation

Series flow. The reference cell of the uv photometer was connected between the gradient chamber and the inlet to pump 2.

Dual columns. An additional column was connected parallel to the analytical column. The effluent from this column was connected to the reference cell of the uv photometer. The effluent from the reference or sample cells was then passed through a bubble-tube flow monitor.

Chemical compensation. The absorbance of the 0.015M acetate buffer was increased by adding small amounts of adenosine, guanosine, toluene, or acetone until the absorbance at 254 nm, measured on a Beckman DBG spectrophotometer, was identical with that of the 6M sodium acetate buffer.

Data acquisition. Since the output of the uv photometer is linear with respect to concentration, the output may be displayed graphically on a

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**Fig. 1. Functional diagram of LCS-1010**
stripchart recorder in the form of typical chromatographic peaks, or fed into an electronic digital integrator or computerized chromatography data system for direct data processing. Chromatography Data System 200 (Varian Aerograph) was used in these studies to evaluate and compare the chromatograms.

**Recorder.** Two different recorders were used satisfactorily during the experiments: a dual-pen Model 20 (Varian Aerograph) and a single pen Model PS01W6A (Texas Instruments, Inc., Houston, Texas 77006). Both recorders record continuously and have adequate filtering (time constant about 1 s) for liquid chromatography.

**Analytical Methodology**

**Sample preparation.** Urine samples were collected from healthy men. The samples were refrigerated at below -5°C between collection and analysis, but were otherwise untreated, except as noted.

**DEAE chromatography.** Some urine samples were prefractionated into an acidic and combined basic-plus-neutral fraction by DEAE-cellulose chromatography by Drs. Linus Pauling and Art Robinson (Stanford University), who used the procedure of Horning (12).

**Cation-exchange prefractionation.** A mixture of whole urine (2.0 ml) buffered at pH 3.25 with 1.0 M KH₂PO₄ (pH 3.25, 0.5 ml) and 0.5 g of AG50X8 per 100 ml was shaken for 5 min. The supernatant fluid was injected directly.

**Solvent extraction.** Buffer solution (0.5 ml) was added to 2.0 ml of whole urine. The resulting mixture was extracted twice with 10-ml portions of chloroform or ethyl acetate. The results are presented in Table 2.

**Controlled diet samples.** Urine samples from patients who had on a synthetic low-residue diet were provided by Drs. Pauling and Robinson. The diet, orange-flavored “Vivonex 100”, is manufactured by Vivonex Corp., Mountain View, Calif. 94305.

**High-performance chromatography.** The urine sample was chromatographed by stopping the eluant flow, decreasing the pressure, and then filling the 200-µl sample loop with urine. The urine sample is then eluted with a linear acetate gradient (flow rate of 8 ml/h), formed by placing 90 ml of the 0.015 M buffer, in the gradient chamber and pumping into it the 6.0 M buffer at a flow rate of 4 ml/h. Column temperature was maintained at 60°C for the entire run.

**Results and Discussion**

**Gradient Programming**

A typical chromatogram from a 100-kg male is shown in Figure 2A. More than 100 peaks were detected in this urine. The analysis took 20 h.

Fig. 2. Effect of gradient program on the chromatography of urine

See Table 1 for conditions. The solid line represents the gradient profile.

Attempting to decrease the analysis time, we investigated the effect of changing the gradient program. The chromatograms in Figures 2A and 2B were obtained with linear gradients. The straight black lines in the Figures depict the shape of the gradient. Details on how the linear, concave, and convex were formed are found in Table 1. In chromatogram 2B, the linear gradient was delayed four hours. During the 4 h, the column was eluted with the dilute component of the gradient (i.e., the 0.015 M buffer). The result was a better resolution of the peaks in the earlier region of the chromatogram. The third chromatogram was obtained by using a concave acetate gradient. This resulted in increased resolution in the region of the chromatogram between the uric and hippuric acid peaks. Chromatogram 2D was obtained by use of a convex acetate gradient, which compressed the chromatogram, with some loss of resolution in the earlier regions, but halved analysis time. These results demonstrate that a faster analysis is practicable if only a few compounds are of interest.

The gradients for chromatograms 2A-2D were generated by varying the instrument parameters of the LCS-1010 (i.e., initial volume, gradient delay,
Table 1. Operating Parameters Required to Produce Various Gradient Profiles*

(See Figure 2 for Results)

<table>
<thead>
<tr>
<th>Type of gradient</th>
<th>Gradient delay, min</th>
<th>Volume in gradient chamber, ml</th>
<th>Flow rate, ml/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Linear</td>
<td>30</td>
<td>90</td>
<td>4</td>
</tr>
<tr>
<td>B. Delayed linear</td>
<td>240</td>
<td>120</td>
<td>4</td>
</tr>
<tr>
<td>C. Concave</td>
<td>30</td>
<td>120</td>
<td>2</td>
</tr>
<tr>
<td>D. Convex</td>
<td>30</td>
<td>50</td>
<td>8</td>
</tr>
</tbody>
</table>

* Buffer reservoirs 1 and 2 contained 6.0M and 0.015M sodium acetate (pH 4.4), respectively. Pressure in each case was \(48 \times 10^4 \) to \(55 \times 10^4\) N/m² (700–800 psi) and temperature was 60°C.

and flow rates of pumps 1 and 2). It is also possible to vary the chemical properties of the dilute and concentrated components of the buffers, such as polarity, ionic strength, pH, and ligand strength. The top chromatogram in Figure 3 was obtained with a linear gradient generated by using the 0.015M buffer and the 6.0M buffer as the dilute and concentrated components of the gradient, respectively. In the bottom chromatogram, the linear gradient started at 4M sodium acetate buffer, pH 4.4. The region in which hippuric acid appeared was still well resolved, but the elution time was decreased by 11 h, to less than 3 h. In like manner, by varying the ionic strengths of the two components of the gradient, it should be possible to optimize any region of the chromatogram with regard to resolution and analysis time. These examples also demonstrate the ease with which the effects of these changes can be measured.

Linear Range—Dual Detectors

When comparing chromatographic profiles, one would prefer that all peaks remain on scale. This is often difficult because of the variable quantities and molar absorptivities of the individual components contained in urine. The problem may be minimized by monitoring the chromatographic column with two uv detectors containing flow cells with different path-lengths, such as 4 and 10 mm. The advantages of this approach are shown in Figure 4. In the chromatogram obtained with the 10-mm flow cell, several of the large peaks are off scale, but the smaller peaks are accentuated. In the chromatogram obtained from the 4-mm flow cell, the small peaks are minimized, but the large peaks are on scale. Thus, the use of two flow cells in series allows one to compare or quantitate all of the chromatographic peaks obtained from a complex mixture.

The continuous traces from a dual-pen recorder were found to provide a good method for simultaneously recording and comparing chromatograms at high and low sensitivities. This effectively increased the dynamic range of the recorder.

Baseline Compensation

The absorbance of 6M sodium acetate at 254 nm is about 0.30. This limits the usable sensitivity of the uv detector because of baseline drift during gradient elution. Most runs involve starting with a solution of 0.015M acetate, which has an absorbance of less than 0.01 at 254 nm.

This problem is often solved by placing the reference cell of the uv photometer between the gradient chamber and the high pressure pump (pump 2, Figure 1). In this way, difference in drift between the reference and sample cells is minimized.

With the LCS-1000, this solution was not completely satisfactory because the small diameter of the cell and tubing (0.020 inches) presented...
sufficient restriction to "starve" pump 2 at high buffer concentrations. Removing bubbles from the reference cell, which was under vacuum, was particularly difficult.

The net absorption increase that results from the increasing concentration of acetate during the gradient might be compensated by adding a small amount of a strongly uv-absorbing cation to the dilute buffer. Because the absorbers of the two solutions were identical, yet no net change in absorbance would then be expected. Unfortunately, no uv-absorbing material was found that was suitable. Both adenosine and guanosine gave extraneous peaks during the chromatogram. Toluene and acetone also gave extraneous peaks, and the solutions were not reproducible in their absorbance, possibly owing to the volatility of the compounds.

The most satisfactory arrangement was to add a second column, parallelizing the analytical column. A needle valve was put in series with the reference column. The effluent from the valve was passed through the reference cell. In this way, the absorbance drift during the run was minimized to $2.4 \times 10^{-4} A$, a 10-fold improvement in the usable sensitivity.

Use of a computerized data-processing system for baseline compensation and data processing was also investigated. The instrument was found to be compatible with the computer. Since the output detector is linear in absorbance, the computer was able to measure the area, which can be converted directly to composition units if the identity of the compound responsible for the peak is known. Since this was not the case, the area of each peak was presented, tabulated by retention time. At present, the longer elution times exceed the program run-times available. Overcoming this limitation will require only minor alterations of the programs.

Programs have been developed that automatically compensate for baseline drift. Since the computer has a much wider dynamic range than the recorder, the computer may be used to compensate for the absorbance of the buffer and also to measure the smaller peaks.

Effect of Diet on Urine Chromatograms

Diet is known to affect urine composition. For this reason, the effect of diet on urinary profile must be ascertained or minimized. Urine samples were collected from subjects on a synthetic, low-residue diet, "Vivonex 100." The effect of diet on a urinary profile is demonstrated in Figure 5. The upper chromatogram was obtained from chromatography of an aliquot of a 24-h urine sample from a typical subject whose diet was unrestricted. The profile changed steadily during three to seven days on the Vivonex diet. The greatest difference is in the 9–14 h region. The profile of the typical subject is characterized by having only the hippuric acid peak in this region, while nine significant peaks appear in the profiles of the diet-controlled samples. Other significant differences were observed at 1.3, 3.7, and 5.5 h of the chromatograms. In addition to these differences, minor ones were also observed in other regions of the chromatograms.

Variation in the chromatographic profile with length of time on the diet is minimized by day 11. Indeed, chromatograms 5C and 5D are qualitatively the same. Thus, it appears that urine composition reaches a steady state by the seventh day. A similar effect of diet was observed by
The chromatogram of white wine shows the results obtained with a dual-pen recorder with different sensitivities. The two recorder amplifiers were connected in parallel. The recording represented by the upper trace is five times more sensitive than that represented by the lower. Trace or weakly absorbing compounds can be more sensitively detected in this way. All the chromatograms were obtained with continuous, rather than point-by-point, recorders, with resulting improved definition of the chromatographic peaks.

Prefractionation before Chromatography

Compounds found in urine range from strongly acidic to basic. As such, it is often advantageous to decrease the number of peaks in the chromatogram by prefractionating the urine before analysis (18). This often removes constituents that interfere with the determination of a particular compound.

Various methods—including solvent extraction, batch ion-exchange and DEAE-cellulose chromatography (14)—are potentially useful for this. For example, Figure 8 compares the chromatogram of whole urine with that for a sample of urine that had been subjected to a batch cation-exchange clean-up (Chromatogram 8B). The amount of material eluting in the early portion of the chromatogram is significantly diminished, permitting a more rapid analysis (chromatogram 8C). Although not as clean a separation as the previously reported DEAE-cellulose chromatograph (14), the saving in time is significant, since the batch ion-exchange takes less than 10 min.

Extraction with chloroform or ethyl acetate also selectively removes some of the constituents in the first part of the chromatogram (Table 2). The relative peak height of peak 47 to peak 17 is un-

Complex Fluids

The methodology developed to analyze body fluids also has useful industrial chemical applications. Figures 6 and 7 show chromatograms of synthetic vanilla and white wine, respectively. These chromatograms are especially useful in the analysis of flavoring components that have a low volatility. For instance, the chromatogram of vanilla indicates that over 52 components are present. Since the chromatographic conditions are mild, and there is no sample preparation, the compounds may be collected and identified with a high degree of confidence.

Young (10). When chromatograms of a patient taking the Vivonex diet with flavoring are compared with the same patient ingesting the unflavored diet, the unusual peaks in the 9–14 h region are not observed. This indicates that the peaks derive from the flavoring agents or their metabolites. Some of these compounds may be unaltered during passage through the body. Thus, it should be possible to monitor the patient’s consumption. In this way, these compounds could serve as internal standards. From this, a complete material balance may be calculated.
changed. This offers the potential of selectively removing interfering compounds.

Instrument Performance

Qualitative identification on the basis of retention times requires that the retention times be reproducible. The higher the precision of the retention times, the greater the certainty of identification on this basis. A previous report described the reproducibility of retention times for 10 peaks, including hippuric and uric acid on a single column (14). In more than 15 chromatograms, the relative standard deviation (cv) in retention times for hippuric and uric acid was 3.0% and 2.8%, respectively (14). The variation for four columns is summarized in Table 3. Retention times for uric acid range from 4.75 and 5.17 h with a relative standard deviation of 3.89%. The corresponding values for hippuric acid range from 11.20 to 11.72 h, with 2.10% relative standard deviation. This high degree of reproducibility gives one confidence that changing columns will not have a major effect on the chromatogram.

We wish to express our appreciation to Dr. Miner Munk for the design of the short-pathlength uv cell and the sample injector, Mr. Robert Koperski for illustrative advice, Mrs. Margaret Hawken for experimental assistance, and to the management of Varian Aerograph for permission to publish this paper.

References

Fig. 8. Effect of batch cation-exchange treatment on the urine profile
Conditions as in Figure 2A