Fractionation of Human Urine by Gel Chromatography

C. A. Burtis, Gerald Goldstein, and Charles D. Scott

High-resolution anion-exchange chromatography of human urine can resolve more than 150 constituents that absorb ultraviolet light. Prefractionation of urine on Sephadex G-10 helped us identify the constituents and simplified the anion-exchange chromatogram. The material associated with the 13 peaks obtained by gel chromatography was pooled and concentrated into six fractions, which were subsequently analyzed by high-resolution anion-exchange chromatography. Each of these fractions contained from 13 to 63 ultraviolet-absorbing constituents.

Development of a high-resolution anion-exchange chromatographic system (the UV-Analyzer) at the Oak Ridge National Laboratory has resulted in the separation of more than 150 constituents from human urine (1-3) that absorb ultraviolet light (UV). Because these constituents are present in relatively small quantities and are eluted in large quantities of acetate buffer, their identification by routine spectral analysis has proved to be difficult. In addition, incomplete resolution frequently causes a particular peak to be composed of several compounds (rather than a single constituent), thus complicating the interpretation of information obtained by mass spectrometry or nuclear magnetic resonance, or infrared and UV spectra. Therefore, to increase resolution and to simplify the anion-exchange chromatograms, we have prefractionated human urine on Sephadex G-10 and chromatographed the resulting gel fractions on the UV-Analyzer.

Separation of compounds by gel chromatography based on molecular size is well documented (4). Also, electrostatic partition and aromatic and hydrophobic adsorption are responsible for many separations obtained by gel chromatography (4-7). These characteristics of the gel matrix have been used to separate nucleic acids (5, 8-12), amino acids (5), vitamins (7), steroids (13-17), and other urinary constituents (18). Since many of the above compounds, in free or conjugated forms (or both), are found in urine, fractionation by gel chromatography before anion-exchange chromatography has proved to be a useful technique for simplifying the chromatogram and increasing the resolution obtained with the UV-Analyzer.

Experimental

Materials and Equipment

UV-Analyzer. A high-pressure system for anion-exchange chromatography was used as previously described (1-2). This system was equipped with an analytical (0.45 × 200 cm) anion-exchange column and a preparative (1.25 × 200 cm) anion-exchange column that were filled with Dowex 1-X8 particles of 5- to 10-μ and 10- to 20-μ diameters, respectively. The column effluent was monitored with a spectrophotometer at two to four wavelengths (250, 260, 280, and 290 nm).

Gel chromatography. Preswollen Sephadex G-10 gel (40-120 μ, Pharmacia Fine Chemicals, Inc., N. Y.), after removal of fines, was packed by gravity into two Pharmacia columns (5.0 × 76 cm and 2.5 × 86 cm) and then equilibrated several hours with distilled water at 25°C. These columns were connected in series with the larger column preceding the smaller one; when not in use, they were equilibrated and stored in 0.2% sodium azide to prevent microbial contamination. The column effluent, which was monitored at a wavelength of 260 nm with a recording Beckman DB spectrophotometer and by a differential refractometer.

From the Oak Ridge National Laboratory, Oak Ridge, Tenn. 37830. Operated for the U.S. Atomic Energy Commission by the Union Carbide Corp.

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(Model R-4, Waters Associates), was collected by a fraction collector. A block diagram of this system is shown in Figure 1.

**Chromatography of urine samples.** Human urine specimens, as received, had been untreated except for freezing and thawing. A 5-ml aliquot of a 24-h composite urine was placed on the gel columns by an injection valve (Chromatronics, Inc.) and then eluted with distilled water at the rate of 2.2 ml/min. The eluate corresponding to the 13 resulting chromatographic peaks was pooled into six fractions as indicated in Figure 2. After concentration to 20 ml in a rotary evaporator at 37°C, each fraction was chromatographed on the preparative column (1.25 × 200 cm) of the UV-Analyzer, as described previously (1–3). When the analytical column (0.45 × 200 cm) was used, a 1-ml urine sample was chromatographed on the gel system, and each of the resulting fractions was concentrated to 2 ml for anion-exchange chromatography.

**Chemical testing.** Individual, as well as pooled fractions obtained from the fractionation of human urine by gel chromatography were subjected to various chemical tests. The individual fractions were concentrated by rotary evaporation when necessary. The methods used in testing are found elsewhere (19, 20).

**Results and Discussion**

Figure 2 illustrates a representative chromatogram obtained by gel chromatography of human urine. Both the upper chromatogram, which shows the change in absorbance at 260 nm, and the lower chromatogram, which shows the change in refractive index, are plotted as a function of elution volume. Thirteen peaks are observed in the UV-chromatogram. The refractive index chromatogram contains four major peaks, the first three

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>Elution volume, a (ml)</th>
<th>K b</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>790</td>
<td>0.10</td>
</tr>
<tr>
<td>II</td>
<td>925</td>
<td>0.27</td>
</tr>
<tr>
<td>III</td>
<td>1065</td>
<td>0.44</td>
</tr>
<tr>
<td>IVa</td>
<td>1180</td>
<td>0.60</td>
</tr>
<tr>
<td>IVb</td>
<td>1350</td>
<td>0.82</td>
</tr>
<tr>
<td>V</td>
<td>1430</td>
<td>0.92</td>
</tr>
<tr>
<td>Vla</td>
<td>1980</td>
<td>1.63</td>
</tr>
<tr>
<td>Vlb</td>
<td>2190</td>
<td>1.91</td>
</tr>
</tbody>
</table>

a Chromatography on a 5.0 × 76 cm column connected in series to a 2.5 × 86 cm Pharmacia column; 40–120 μm particles of Sephadex G-10 gel; temperature, 20°C; eluant, distilled water, 2.2 ml/min; sample, 5 ml of human urine.

b Calculated from the equation $K_i = (V_e - V_0)/V_e$, $V_e =$ elution volume, $V_0 =$ void volume (714 ml), and $V_i =$ inner volume (75 ml, calculated). $V_i$ estimated from the equation $V_i = (V_e - V_0)/[(1 + W_r)\, d]$ where $V_e =$ total column volume (1920 ml), $W_r =$ water regain of gel (1.0) (g), and $d =$ density of swollen gel (1.24) (g).

c Values represent the mean ± a relative standard error of 3–5% for 8 chromatographic runs.
correspond to UV peaks. The fourth refractive index peak corresponds to material with little UV absorbance; however, this fraction was highly ninhydrin-positive. Urea, which elutes at this time, probably accounts for the strong ninhydrin and refractive index responses associated with low UV absorption.

The partition coefficients \(K_d\) for the six pooled fractions are shown in Table 1. These values are useful for determining the positions at which classes of compounds are eluted from the gel column. Theoretically, compounds that are completely excluded from the gel matrix have the \(K_d\) value of 0. Compounds having partial access to the gel should have \(K_d\) values greater than 0 but less than 1.0. Compounds having complete access to the interior of the gel particles have a \(K_d\) value of 1.0, whereas compounds that interact with or are adsorbed by, the gel matrix will have \(K_d\) values greater than 1.0. Actually, the upper limit of 1.0 is high, since part of the volume enclosed by a gel particle \(V_o\) is composed of bound water of hydration and is inaccessible to solute molecules. This is particularly true for the denser gels and for Sephadex G-10; Janson (7) has estimated a \(K_d\) value of 0.75 for compounds having complete access. If this value is used as a lower limit, it is evident that fractions IVb, V, VIa, and VIb have interacted with, or have been adsorbed by, the gel matrix. Such behavior is known to be typical of aromatic and heterocyclic compounds (7); thus it is possible that these types of compounds are present in the last three gel fractions.

Chemical testing of the eluate is also useful for establishing the positions at which classes of compounds are eluted from the gel column. Tests with ninhydrin reagent showed that ninhydrin-positive compounds are present in fractions I, III, and IV, and in the peak eluted between fractions V and VI (see Figure 2). As discussed previously, the elution position of urea coincides with this peak, and probably accounts for the strong ninhydrin and refractive index responses and the weak UV absorption. Tests with phosphotungstic acid indicated that uric acid was eluted in gel fraction IVb. This was confirmed when the peak corresponding to uric acid was observed in the chromatogram obtained by ion-exchange chromatography of gel fraction IV. Peaks giving tests for ribose and hexoses were observed in fraction IV.
In regard to inorganic anions, phosphates were eluted in fraction I and chlorides in fraction IV. [The separation of anions by gel chromatography has been reported previously by Egan (21).] Tests with ferric chloride and other tests for the presence of aromatic compounds were negative. However, since peaks corresponding to aromatic compounds were later identified in the anion-exchange chromatogram obtained from gel fraction IV, we conclude that the chemical tests for these compounds lack sufficient sensitivity under our conditions. Colorimetric tests for steroid and steroid conjugates were also negative; again, this was probably because of the low sensitivity of the tests in relation to the concentration of these substances in the urine.

In an earlier paper (3), we reported the separation of more than 100 UV-absorbing urinary constituents from human urine by anion-exchange chromatography. A chromatogram of such a separation is shown in Figure 3. This chromatogram was obtained from 2 ml of human urine in an analytical (0.45 × 200 cm) column as previously described (3).

To obtain larger samples for identification purposes, we have chromatographed 20 ml of human urine on a preparative (1.25 × 200 cm) column. The results of this separation are shown in the top chromatogram of Figure 4. Since larger resin particles are used in the preparative column, resolution is decreased. Prefractionation of urine by gel chromatography subsequent to anion-exchange chromatography allows even larger amounts of urine to be separated.

Results of anion-exchange chromatography of the gel fractions are shown in Figure 4. The upper chromatogram was obtained by chromatographing 20 ml of human urine, using the preparative column of the UV-Analyzer. Eighty peaks were resolved. The remaining six chromatograms show that gel fractions I, II, III, IV, V, and VI were resolved into 18, 30, 13, 63, 12, and 17 peaks, respectively.

The chromatogram from gel fraction IV was of interest since material associated with a large number of peaks was eluted in 17 to 35 h. We had observed previously that most of the aromatic acids were eluted in this region (3). Because the $K_d$ value indicates that the compounds in fraction IV interact with the gel matrix and because aromatic compounds show a similar response on a gel column, we can speculate that gel fraction IV contains a number of the aromatic acid type of compounds in urine. Thus, gel chromatography of urine enables the separation of the acid components of urine from other urinary constituents.

Uric acid elutes in 16.5 h and hippuric acid in 23 h in the analytical anion-exchange chromatogram (Figure 3) (3). Both of these compounds...
are contained in gel fraction IV (Figure 4). Using the analytical column of the UV-Analyzer, we were able to determine that hippuric acid was eluted in the first peak (IVA) and uric acid was eluted in the second peak (IVb) of gel fraction IV. The same elution sequence has been reported by Sinha and Gabrielli (18), with 0.1M phosphate buffer, pH 7.0, as the eluting solvent. Differences in the elution sequence of these two compounds may result with different elution solvents.

Eaker and Porath (6) reported that increasing the ionic strength of a solvent will affect elution position of compounds on a gel column by decreasing their effective molecular size or by increasing the volume of gel accessible to the compound.

We eluted with water because it can easily be removed by evaporation when preparing the gel fractions for anion-exchange chromatography. The increased resolution of hippuric and uric acid obtained with the phosphate buffer as compared with water was not necessary; these two compounds are well separated by anion-exchange chromatography.

Chromatography of gel fraction IV on the UV-Analyzer shows that this fraction contains numerous other UV-absorbing compounds in addition to uric and hippuric acids. Therefore, if these two acids in fraction IV were to be estimated by spectrometric assay methods, the results would be erroneously high because of the contributions of the other UV-absorbing constituents. Gel chromatography is useful for class separations but not for the separation of individual components from such a complex mixture as urine. However, peaks in several previously unresolved areas of the anion-exchange chromatogram can be resolved if the urine is prefractionated by gel chromatography and the resulting fractions are then chromatographed on the UV-Analyzer. For example, the peak corresponding to uric acid in a chromatogram of normal urine is found to contain as many as nine compounds when the urine is pretreated. Thus, this technique could be used to
separate and detect smaller peaks, and individual constituents could then be isolated and analyzed by other methods.

Refractionation improves resolution of constituents eluted during the first 6 h of anion-exchange chromatography of normal urine. Most of these constituents are thought to be either neutral or basic since many neutral and basic compounds have been shown to elute in this region of the chromatogram (3). Improved resolution is observed in fractions II, III, and IV (Figure 3), which contain single peaks instead of the usual large unresolved areas. An increase in resolution is also noted in the 6- to 7-h and the 11- to 12-h eluates. The 6- to 7-h eluate is resolved into single peaks in gel fractions II, IV, and V, and the 11- to 12-h eluate is resolved into single peaks in gel fractions I, II, IV, and V.

Our results have confirmed that human urine is a complex biological fluid. The anion-exchange chromatography of urine on the UV-Analyzer has generated a difficult problem with regard to the identification of the newly recognized peaks. The prefractionation of urine by gel chromatography has proved to be a useful technique for separating classes of urinary constituents. It has provided increased resolution of peaks that occur in the 0- to 6-h, 6- to 7-h, and the 11- to 12-h eluates. We are now attempting to analyze these resolved peaks by routine spectrometer analysis.

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References


