

Determination of Free and Conjugated Bile Acids as Ultraviolet-Absorbing Ion Pairs by Reverse-Phase High-Performance Liquid Chromatography

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A method for the determination of free and conjugated bile acids as uv-absorbing ion pairs was developed. Ultraviolet photometric detection was more sensitive than differential refractometer detection. Improved resolution of positional isomers was also achieved. Distinctions were made between free and conjugated bile acids and between tauro- and glyco-conjugated bile acids. This was accomplished by adjusting the pH of the mobile phase to selectively form ion pairs.

A number of methods for improving the resolution and detection of free (1,2) and conjugated (3,4) bile acids have been reported. These require the preparation of uv-absorbing phenacyl ester derivatives (2) or uv-absorbance detection in the 200-nm region (1,3,4). Ultraviolet detection at low wavelengths offers considerably higher sensitivity particularly for bile acids which are conjugated with amino acids, such as glycine and taurine, than for the free bile acids (1).

A method for the determination of free and conjugated bile acids as uv-absorbing ion pairs is discussed in the present paper. Of particular interest is the improved resolution obtained for positional isomers of bile acids as a result of ion pairing and the effect of pH on the formation of uv-absorbing ion pairs.

MATERIALS AND METHODS

Apparatus. The hplc¹ system consisted of a minipump (Milton Roy, Riviera Beach,

Fla.),² a loop injector (Rheodyne, Berkeley, Calif.), and an analytical column (30 cm × 4.6 mm i.d.) packed with 10 μm Chromegabond-C₁₈ (E. S. Industries, Marlton, N. J). The detectors were a differential refractometer, model R-401, and a uv-photometer (254 nm), model 440 (Waters Associates, Milford, Mass.). All solvents were filtered through a 0.45-μm Millipore filter (Millipore, Bedford, Mass.) before use.

Operating conditions. The counterion, Hyamine 1622 (diisobutylethoxyethyl-dimethylbenzyl ammonium chloride monohydrate), was added directly to the mobile phase, which contained 0.002 or 0.007 M H₃PO₄, and the pH was adjusted to the desired value with 10% (v/v) NH₄OH). The mobile phase was then pumped through the column for 0.5 h until equilibration was achieved. Solutions of the bile acid samples, in the mobile phase, were prepared at concentrations of 0.1% (w/v). Samples (20 μl) were injected onto the column, and separations were monitored by the uv photometer

¹ Abbreviations used: hplc, high-performance liquid chromatography; RI, differential refractometer; AUFS, absorbance units full scale; V₀, void volume; V_R, retention volume.

² Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

and the RI detector, arranged in series, at sensitivities of 0.05 AUFS and 4 \times , respectively. When not in use, the counterion was purged from the column with several volumes of aqueous alcohol to prolong the life of the column.

Reagents. Free bile acids (ICN Pharmaceuticals Inc., Cleveland, Ohio), conjugated bile acids (Sigma Chemical Co., St. Louis, Mo.), and Hyamine 1622 (Rohm & Haas, Philadelphia, Pa.) were used without further purification.

RESULTS AND DISCUSSION

Separation of bile acids and their conjugates as uv-absorbing ion pairs by reverse-phase hplc often provides better resolution and more versatility than conventional re-

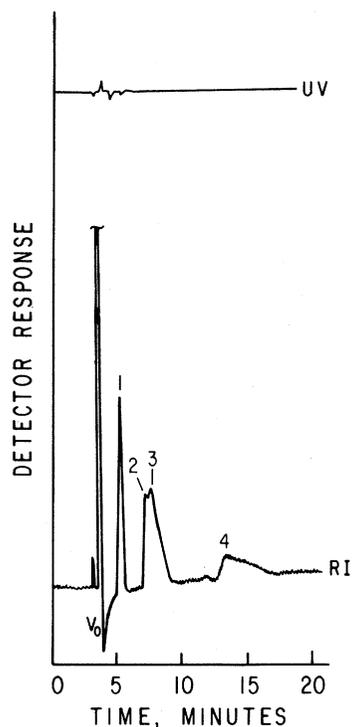


FIG. 1. Separation of free bile acids. Mobile phase (80/20) methanol/water containing 0.002 M $(\text{NH}_4)_2\text{HPO}_4$, pH = 7.5. Flow, 1 ml/min. Peak 1, cholic acid; peak 2, chenodeoxycholic acid; peak 3, deoxycholic acid; peak 4, lithocholic acid.

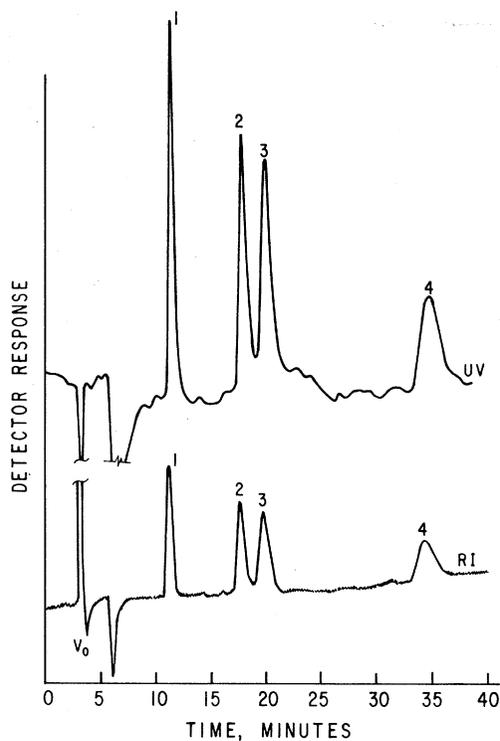


FIG. 2. Effect of Hyamine 1622 on bile acid separation. Mobile phase (75/25) methanol/water containing 0.09% Hyamine 1622 (w/v), 0.002 M $(\text{NH}_4)_2\text{HPO}_4$, pH = 7.5. Flow, 2 ml/min. Peaks in same order as in Fig. 1.

verse-phase separations. For example, Fig. 1 demonstrates the poor separation for a mixture of cholic acid 1, chenodeoxycholic acid 2, deoxycholic acid 3, and lithocholic acid 4. RI detection was used to monitor this separation. Addition of the uv absorbing counterion, Hyamine 1622, to the mobile phase improved the separation of these four bile acids, especially the positional isomers chenodeoxycholic acid 2 and deoxycholic acid 3 (Fig. 2). Since free bile acids exhibit no uv absorbance at 254 nm, addition of the counterion permitted their detection as uv absorbing ion pairs by the uv photometer. Sensitivity was improved and detection limits for free bile acids were 1 μg . The negative peak after V_0 resulted from the lower concentration of counterion in the injected

sample solution due to pairing of the counterion with the bile acid. Similar separations were obtained for separate mixtures of the tauro- and glyco-conjugated bile acids with this technique.

Differentiation of free bile acids from the conjugated bile acids or the tauro-conjugates from the glyco-conjugated bile acids by selective formation of ion pairs demonstrates the versatility of this method. Table 1 lists the V_R of free and conjugated bile acids obtained with mobile phases buffered to pH

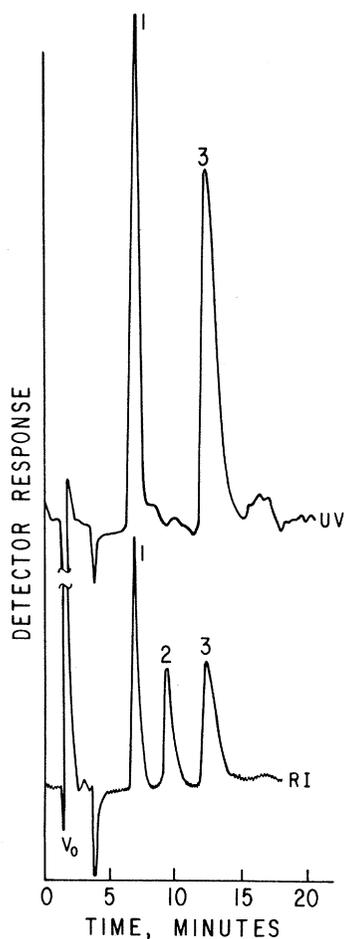


FIG. 3. Separation of conjugated cholic acid derivatives as uv-absorbing ion pairs. Conditions same as in Fig. 2 except 0.002 M $\text{NH}_4\text{H}_2\text{PO}_4$, pH = 4.7. Peak 1, glycocholic acid; peak 2, cholic acid; peak 3, taurocholic acid.

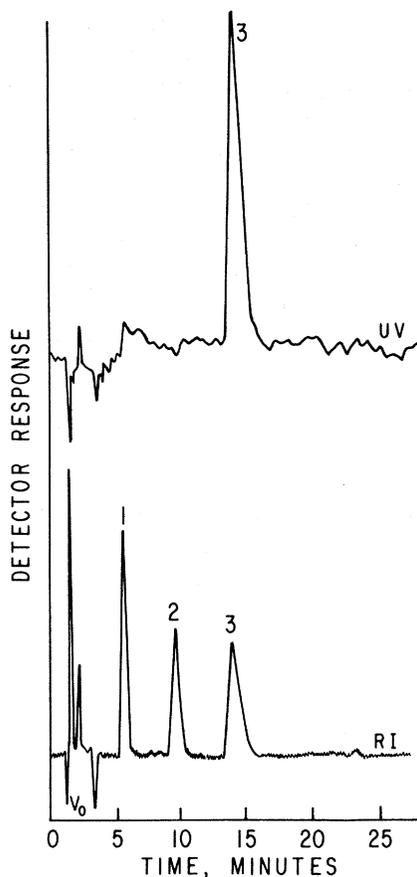


FIG. 4. Separation of taurocholic acid as uv-absorbing ion pair. Conditions same as in Fig. 2 except 0.007 M H_3PO_4 , pH = 3.3. Peak order same as in Fig. 3.

7.5, 4.7, and 3.3. Both free and conjugated bile acids exhibited limited solubility in the mobile phase and were poorly retained on the column when Hyamine 1622 was not included in the mobile phase. Upon addition of Hyamine 1622 to the mobile phase and buffering to pH 7.5, all the bile acids formed ion pairs which resulted in their increased solubility in the stationary phase, hence, greater retention. When the pH of the mobile phase was adjusted to 4.7, the conjugated bile acids were detected as uv-absorbing ion pairs and the free bile acids remained unpaired. This permitted differentiation between the free and conjugated bile acids. At pH 3.3 differentiation between the glyco- and

TABLE 1
EFFECT OF pH ON ION PAIRING OF BILE ACIDS

Bile acid	V_R (ml)			
	pH = 7.5	0.09% (w/v) Hyamine 1622		
		pH = 7.5	pH = 4.7	pH = 3.3
Cholic	6.0	24.4*	18.4	18.0
Chenodeoxycholic	**	45.2*	37.2	36.8
Deoxycholic	**	54.0*	40.4	40.4
Glycocholic	4.8	24.0*	14.4*	10.8
Glycochenodeoxycholic	—	42.0*	22.8*	18.4
Glycodeoxycholic	6.8	49.2*	26.4*	21.2
Taurocholic	4.4	24.4*	21.6*	24.0*
Taurochenodeoxycholic	—	42.0*	39.6*	40.4*
Taurodeoxycholic	6.4	50.8*	47.2*	46.8*

* Detected as uv-absorbing ion pairs by the RI and uv detectors.

** Insufficient solubility in mobile phase.

tauro-conjugates was made, since only the tauro-conjugated bile acids formed uv-absorbing ion pairs. Selective ion pairings for cholic acid derivatives at pH 4.7 and 3.3 are shown in Figs. 3 and 4, respectively. At pH 4.7 conjugated cholic acid derivatives 1 and 3 were distinguished from free cholic acid 2 as ion pairs (Fig. 3). At pH 3.3 only taurocholic acid 3 formed a uv-absorbing ion pair (Fig. 4).

For simplicity, this study emphasized the use of Hyamine 1622 as the counterion. Other counterions can be used to modify V_R by increasing or decreasing the solubility of the ion pair in the stationary phase. We observed that peak symmetry and resolution improved for those counterions which ex-

hibited some retention on the column under the same chromatographic condition for which the ion pair separations were carried out.

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