Journal of Chromatography, 194 (1980) 80–84
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,733

Note

Separation of steroid glucuronides by reversed-phase liquid column chromatography

JÖRGEN HERMANSSON

National Board of Health and Welfare, Department of Drugs, Box 607, 751 25 Uppsala (Sweden)

(First received December 12th, 1979; revised manuscript received January 31st, 1980)

During pregnancy, estrogens conjugated with glucuronic acid, particularly estriol conjugates, dominate the steroids present in urine. A decrease in the excretion level of estriol indicates a possible malfunction of the placenta. These metabolites are measured routinely by a method involving extraction and derivatization by the Kober reaction followed by spectrophotometric measurements[1].

Huber *et al.*[2] and Dolphin and Pergande[3,4] have used high-performance liquid chromatography (HPLC) for the isolation of estrogen conjugates in urine after hydrolysis and extraction of the liberated aglucone. A drawback of methods involving hydrolysis is that the identity of the individual conjugates is destroyed. In order to avoid this, Van der Wal and Huber[5] used XAD-2 extraction, followed by isolation on an anion-exchange column.

The present paper describes studies of reversed-phase column chromatography of glucuronides of estrone, estradiol and estriol and a method for the isolation of estriol 16α-glucuronide from untreated pregnancy urine.

EXPERIMENTAL

Apparatus

The pump was an Altex Model 100 solvent delivery system and the detector was a Waters Model 440 with an 12.5-μl cell and wavelength of 280 nm. The columns were made of 316 stainless steel with a polished surface, equipped with Swagelok connectors and stainless-steel frits (2 μm). The column dimensions were 150 × 4.5 mm. A high-pressure injection port was used (Rheodyne, 5000 p.s.i.) with a 20-μl loop. Solvent reservoir and column were thermostated by a water-bath (HETO Type 02 PT 923 TC; Birkeröd, Denmark). The pH was measured with an Orion Research Model 801 A/digital meter, equipped with an Ingold Type 401 combined electrode. The gas chromatography–mass spectrometry (GC–MS) system was an LKB Model 2091 with electron impact (EI) ionisation. The GC column was 1.5 m of 3% SE-30.

Chemicals and reagents

1-Pentanol was of ACS reagent quality (Fisher Scientific, Pittsburgh, PA, U.S.A.). The steroid glucuronides were obtained from Sigma (St. Louis, MO, U.S.A.) and used without further purification. All other substances used were of analytical

0021-9673/80/0000–0000/$02.25 © 1980 Elsevier Scientific Publishing Company

grade. The chromatographic support was LiChrosorb RP-8 with a mean particle diameter of 5 μm (E. Merck, Darmstadt, G.F.R.). Buffers had an ionic strength of 0.1.

Column preparation

LiChrosorb RP-8 was packed by a balanced-density slurry technique[6], suspended in carbon tetrachloride–tetrabromoethane–dioxane (1:1:1). After packing, the column was washed with hexane and acetone. The support was coated by passing the mobile phase through it until a test sample gave a constant capacity factor.

Chromatographic technique

The mobile phase reservoir was kept in a water-bath at 25 $\pm$ 0.1 °C. The column was kept in a water jacket with circulating water of the same temperature. Tubing and the injection port were insulated to avoid temperature changes. The mobile phase was prepared by saturating the phosphate buffer with 1-pentanol in a separating funnel. When a lower content of 1-pentanol was used, the saturated solution was diluted with phosphate buffer to the appropriate 1-pentanol concentration. The volume of the mobile phase was determined by injection of potassium dichromate. The samples were dissolved in the mobile phase.

Isolation and GC–MS identification of estriol 16α-glucuronide

The untreated pregnancy urine was injected directly on the reversed-phase column, and a fraction containing the peak with a capacity factor equal to that of estriol 16α-glucuronide was collected. Fractions from eight injections were pooled, acidified with hydrochloric acid to a final concentration of 2 *M* and heated for 90 min at 100 °C. The solution was extracted twice with an equal volume of water-saturated ethyl acetate. The combined extracts were evaporated to dryness.

The sample was dissolved in 20 μl pyridine, and 100 μl acetic anhydride were added. The reaction mixture was then kept in an ultrasonic bath for 5 min, heated at 80 °C for 30 min, evaporated to dryness and the residue dissolved in ethyl acetate. A 2-μl volume of the solution was injected in the GC–MS apparatus. The gas chromatograph was operated at a column temperature of 220 °C and an injector temperature of 240 °C. Helium at a flow-rate of 30 ml/min was used as carrier gas. The ion source of the mass spectrometer was operated at 260 °C with an electron energy of 70 eV.

RESULTS AND DISCUSSION

Regulation of the retention

The steroid glucuronides are weak acids with $pK_a \approx 3.5$[7]. They can be separated in reversed-phase systems with LiChrosorb RP-8 as solid phase and phosphate buffer pH 6.5, containing 1.25–2.5% of 1-pentanol as mobile phase. The capacity ratio can be regulated within rather wide limits by changes of the 1-pentanol concentration in the mobile phase, as demonstrated in Fig. 1.

In the chromatographic system used, 1-pentanol is adsorbed on the hydrophobic solid phase. If the adsorbed 1-pentanol constitutes a liquid stationary phase, the capacity ratio of the glucuronides, k'_X, is given by:

$$k'_X = (V_s/V_m) \times D = (D \times V_t/V_m) - D \quad (1)$$

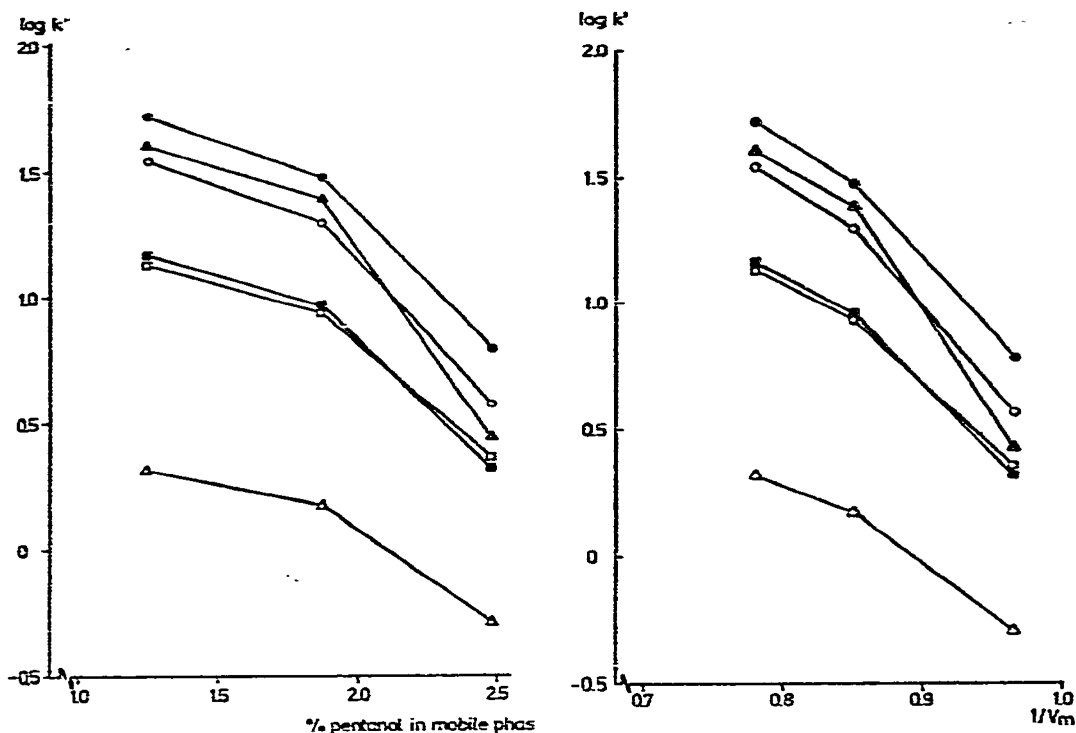

Fig. 1. Regulation of the capacity factor by addition of 1-pentanol. Mobile phase: phosphate buffer pH 6.5 + 1-pentanol. Flow-rate: 2.2 mm/sec. Support: LiChrosorb RP-8 (5 μm). Samples: ●, estradiol 17β-glucuronide; ▲, estrone 3β-glucuronide; ○, estradiol 3β-glucuronide; ■, estriol 16α-glucuronide; □, estriol 17β-glucuronide; △, estriol 3β-glucuronide.

where D is the distribution ratio between stationary and mobile liquid phase, V_s and V_m are the volumes of the stationary and the mobile liquid phases, respectively, and V_t the sum of V_s and V_m. With increasing content of pentanol in the mobile phase, V_s increases[8] and V_m decreases. If eqn. 1 is valid and D is constant, the capacity factor should increase with increasing $1/V_m$. Fig. 1 shows, however, that there is a strong decrease of k' with increasing $1/V_m$. This indicates that the retention is mainly due to adsorption, which is in accordance with observations made for hydrophobic acids, amines and steroid glucuronides[8–10]. Increase of the pentanol content in the mobile phase also gives rise to changes in the separation selectivity, and there are even changes in retention order in some cases.

Separation efficiency

The separation efficiency is good in the systems containing 1.25 and 1.9% of pentanol, the reduced plate height at a flow-rate of 2.2 mm/sec being 5–9 for all compounds, except estriol 3β-glucuronide, where a reduced plate height of about 15 was obtained. Increase of the pentanol content to 2.5% strongly reduces the separation efficiency. This is illustrated in Fig. 2, which shows a drastic increase of the asymmetry of the peaks when the pentanol content is increased from 1.9 to 2.5%.

The sepation efficiency is fairly independent of the capacity ratio at $k' > 8$,

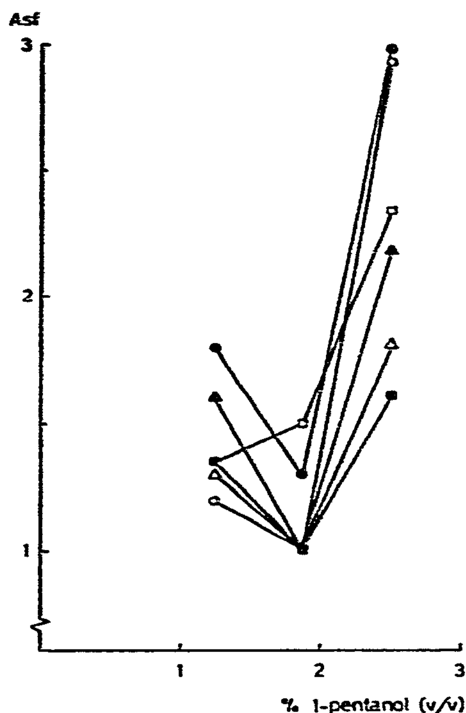

Fig. 2. Asymmetry factors obtained at different concentrations of 1-pentanol. For chromatographic data and samples see Fig. 1. Asf = back part of W_b/front part of W_b. Front and back part of W_b are calculated at the base of the peak, by drawing two tangents of the peak and a perpendicular from the formed vortex.

as demonstrated in Fig. 3. However, a considerable increase in the reduced plate height is observed when the capacity factor approaches unity, which may indicate influence of mass transfer in the stationary phase.

Chromatographic isolation of estriol conjugate from human urine

The chromatographic system could be used for isolation of estriol 16α-glucuronide from untreated human urine. A chromatogram obtained after injection of 20 μl of urine is shown in Fig. 4. Mobile phases containing 1.25% and 1.9% of pentanol gave similar chromatograms, but the retention of the glucuronide was somewhat higher at the lower 1-pentanol content.

The chromatographic systems showed good stability, capacity factors and separation efficiency remaining almost constant after injection of 50 urinary samples.

Identification the metabolite

The identity of the compound in peak 1 (Fig. 4) was established by GC–MS, as described under Experimental. The procedure involved hydrolysis with hydrochloric acid, extraction of the hydrolysis product with ethyl acetate and acetylation with acetic anhydride before injection into the GC–MS system. A solution of estriol in pyridine, treated with acetic acid anhydride in the same manner, was used as reference. The two mass spectra coincided. This indicates that the aglucone portion of the isolated compound is estriol.

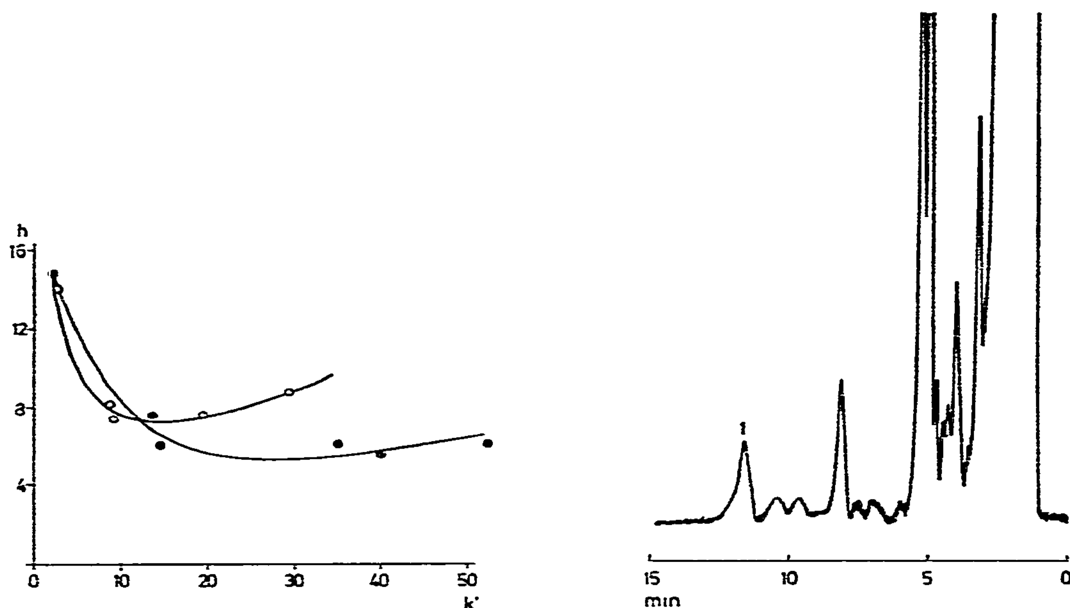

Fig. 3. Relation between the capacity factor and the separation efficiency. For chromatographic data and samples see Fig. 1.
Concentration of 1-pentanol (% v/v): ●, 1.25; ○, 1.9.

Fig. 4. Isolation of conjugated estriol from urine. For chromatographic data see Fig. 1, a.u.f.s. = 0.005. Concentration of 1-pentanol in the mobile phase; 1.9%.
Peak 1 = estriol-16-glucuronide.

Pregnancy urine spiked with estriol 17α-glucuronide gave rise to a double peak. The addition of estriol 16β-glucuronide gave only one peak with a larger area than that obtained with unspiked urine. This indicates that the isolated metabolite is estriol conjugated at C-16 with glucuronic acid.

ACKNOWLEDGEMENT

I am very grateful to Professor Göran Schill for valuable criticism of the manuscript.

REFERENCES

1 S. Kober, *Biochem. Z.*, 239 (1931) 406.
2 J. F. K. Huber, J. A. R. J. Hulsman and C. A. M. Meijers, *J. Chromatogr.*, 62 (1971) 79.
3 R. J. Dolphin, *J. Chromatogr.*, 83 (1973) 421.
4 R. J. Dolphin and P. J. Pergande, *J. Chromatogr.*, 143 (1977) 267.
5 Sj. van der Wal and J. F. K. Huber, *J. Chromatogr.*, 135 (1977) 305.
6 R. E. Majors, *Anal. Chem.*, 44 (1972) 1722.
7 R. T. Williams, *Detoxication Mechanisms*, Wiley, New York, 2nd ed., 1959, p. 729.
8 K.-G. Wahlund and I. Beijersten, *J. Chromatogr.*, 149 (1978) 313.
9 K.-G. Wahlund and A. Sokolowski, *J. Chromatogr.*, 151 (1978) 299.
10 J. Hermansson, *J. Chromatogr.*, 152 (1978) 437.