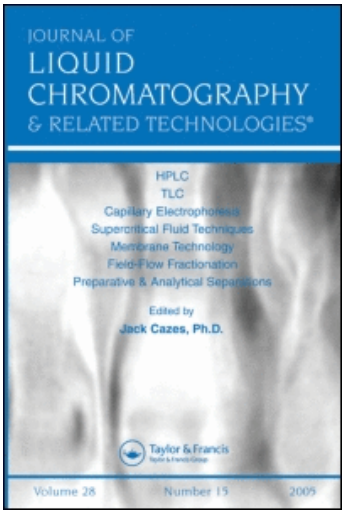

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STUDIES ON STEROIDS CLXXVIII. SEPARATION OF ESTROGEN GLUCURONIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Separation of monoglucuronides of estrone, estradiol, estriol and 16-epiestriol by high-performance liquid chromatography on a reversed-phase column has been carried out. The effects of pH and salt concentration of a mobile phase on the k' value were investigated with a TSK GEL LS-410 ODS-SIL column. Isomeric monoglucuronides of estriol and 16-epiestriol were distinctly separated on this column when 0.7% disodium hydrogen phosphate (pH 3.0)/tetrahydrofuran was used as a mobile phase.

INTRODUCTION

In recent years, considerable attentions have been drawn to the metabolic significance and physiological role of estrogen conjugates in the feto-placental unit. These metabolites are determined by spectrophotometry (1), gas chromatography-mass spectrometry (2,3), or radioimmunoassay (4). Several papers have been recently published dealing with the method for quantitative determination of estrogens in biological fluids by

high-performance liquid chromatography (HPLC) which involves prior hydrolysis and/or solvolysis of the conjugates (5-8). These methods, however, have inevitable disadvantages, the lack of reliability on analytical results and the loss of information about the conjugated form. This paper describes the separation of monoglucuronides of classical estrogens, i.e. estrone, estradiol, estriol and 16-epiestriol, by HPLC.

EXPERIMENTAL

Materials

Estrogen glucuronides were synthesized in these laboratories by the methods previously reported (9). All the reagents used were of analytical reagent grade. Solvents were purified by distillation prior to use.

Instruments

The apparatus used for this work was a Toyo Soda HLC-803A high-performance liquid chromatograph (Toyo Soda Co., Tokyo) equipped with a Model SF-770 ultraviolet (UV) detector monitoring the absorbance at 280 nm. A TSK GEL LS-410 ODS-SIL column (30 cm x 0.4 cm i.d.) (Toyo Soda Co.) was employed under ambient conditions. The pH of the mobile phase was adjusted with phosphoric acid.

RESULTS AND DISCUSSION

The separation of estrogen glucuronides by HPLC has been previously reported by several groups. However, the resolution of estriol 16-glucuronide and estriol 17-glucuronide still remains unsatisfactory (10-12). An initial effort was therefore directed to the separation of these two conjugates. Among the typical columns suitable for the polar compounds, TSK GEL LS-410 ODS-SIL was chosen for this purpose.

The effect of pH of a mobile phase on the capacity ratio (k') was investigated with the 0.7% disodium hydrogen phosphate /tetrahydrofuran system. The k' values of typical estrogen glucuronides relative to estriol 17-glucuronide were plotted against pH of the mobile phase (Figure 1). The close similarity in the chromatographic behaviors was observed between estriol 16-glucuronide and estriol 17-glucuronide in the pH range 5.0 to 7.0. The relative k' value of estriol 16-glucuronide increased with decreasing pH value from 5.0 to 3.0. A similar relation was also found between 16-epiestriol 16-glucuronide and 16-epiestriol 17-glucuronide. This phenomenon can be explained

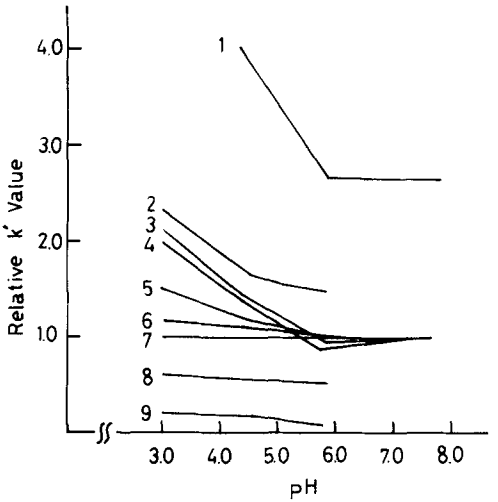

FIGURE 1 Effect of pH on Relative k' Value.
1, Estradiol 17-glucuronide; 2, 16-epiestriol 16-glucuronide; 3, estradiol 3-glucuronide; 4, estrone 3-glucuronide; 5, 16-epiestriol 17-glucuronide; 6, estriol 16-glucuronide; 7, estriol 17-glucuronide; 8, 16-epiestriol 3-glucuronide; 9, estriol 3-glucuronide. Conditions: column, TSK GEL LS-410 ODS SIL; mobile phase, 0.7% Na_2HPO_4/tetrahydrofuran (6:1), 2 ml/min; detection, UV 280 nm.

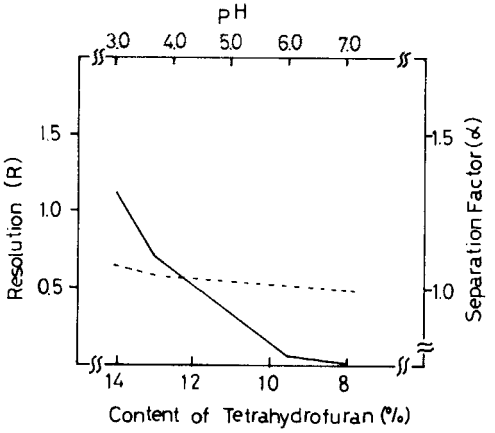

FIGURE 2 Effect of pH and Composition of Mobile Phase on the Separation of Estriol 16- and 17-Glucuronides. ——:Resolution, ---:separation factor. Conditions: mobile phase, 0.7% Na_2HPO_4/tetrahydrofuran. Other conditions were as in Fig. 1.

in terms of dissociation of steroid glucuronides having a glucuronic acid moiety (pK 3.20) in acidic medium where undissociated species are dominant. The effects of pH and composition of the mobile phase on the resolution of estriol 16-glucuronide and estriol 17-glucuronide were also observed at the constant k' value (Figure 2). The separation factor (α) was not significantly influenced, while the resolution (R) was improved with decreasing pH and increasing content of tetrahydrofuran of the mobile phase. These data suggested that the separation of these compounds is considerably dependent upon the pH value of the mobile phase.

The effect of salt concentration on the retention value was then examined by using aqueous disodium hydrogen phosphate (pH 3.0)/tetrahydrofuran as a mobile phase. The k' values of three glucuronides relative to the corresponding value of each glucuronide obtained with 0.4% disodium hydrogen phosphate (pH

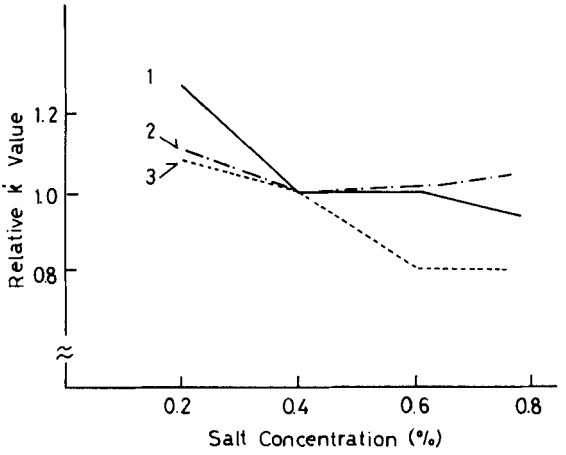

FIGURE 3 Effect of Salt Concentration of Mobile Phase on Relative k' Value of Estriol Monoglucuronides. 1, Estriol 17-glucuronide; 2, estriol 16-glucuronide, 3, estriol 3-glucuronide. Conditions: mobile phase, Na_2HPO_4 (pH 3.0)/ tetrahydrofuran (6:1). Other conditions were as in Figure 1.

3.0) were determined (Figure 3). No remarkable difference in the retention value was found between estriol 16-glucuronide and estriol 17-glucuronide in the concentration range 0.4-0.6%, but the relative k' value of estriol 17-glucuronide slightly decreased with increasing salt concentration from 0.6 to 0.8%. Also estriol 3-glucuronide showed a slight decrease in the k' value with increasing salt concentration.

Based upon these data, 0.7% disodium hydrogen phosphate (pH 3.0)/tetrahydrofuran (6:1) was chosen as a suitable mobile phase. A synthetic mixture of 3-, 16- and 17-glucuronides of estriol and 16-epiestriol were efficiently separated by using the above solvent system (Figure 4). To the best of our knowledge this is the first reported complete separation of isomeric monoglucuronides of estriol and 16-epiestriol by HPLC. Under this condition estradiol 17-glucuronide required a long

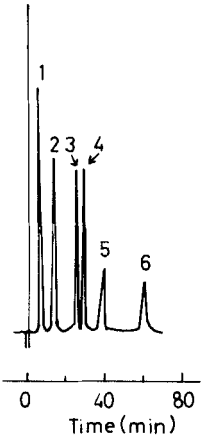

FIGURE 4 Separation of a Synthetic Mixture of Estrogen Monoglucuronides.
1, Estriol 3-glucuronide; 2, 16-epiestriol 3-glucuronide; 3, estriol 17-glucuronide; 4, estriol 16-glucuronide; 5, 16-epiestriol 17-glucuronide; 6, 16-epiestriol 16-glucuronide.
Conditions: mobile phase, 0.7% Na_2HPO_4 (pH 3.0)/tetrahydrofuran (6:1), 1.5 ml/min. Other conditions were as in Figure 1.

time for elution (Figure. 1), but this problem was overcome by using 0.4% disodium hydrogen phosphate (pH 7.5)/methanol (2:1) as a mobile phase.

The application of the present method to the separation of estriol 16-glucuronide and estriol 17-glucuronide in rat bile and human pregnancy urine (13) will be reported elsewhere in the near future.

ACKNOWLEDGMENTS

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