Application of β-Cyclodextrin for the Analysis of Estrogenic Steroids in Human Urine by High-Performance Liquid Chromatography

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Summary
A procedure is described for simultaneous determination of estriol, estrone and 17β-estradiol in human urine. After acid hydrolysis of the sulphate conjugates, the estrogens were extracted into diethyl ether. The ether extracts were concentrated and applied directly to an HPLC column. Using a 25 cm C-18 column and acetonitrile-water modified by the addition of β-cyclodextrin as mobile phase, the separation of estriol, estradiol and estrone was achieved within 20 minutes. The extraction of estrogens from the biological matrix is excellent. Estrogens were detected using a UV-detector (280 nm) or a spectrofluorimetric detector (λex = 280 nm, λem = 312 nm). The latter detection system has been used for the determination of estrogens in the urine of non-pregnant women. The procedure is simple and can be used in clinical practice.

Introduction
Estrogens are of clinical and analytical interest for many reasons. During pregnancy, their urinary concentration in patients with gestosis are lower than those in normal pregnant subjects [1]. Estrogens play an important role in the diagnosis of placental sulfatase deficiency. A high ratio of 3β, 16α-dihydroxy-5-androsten-17-one and androstenetriol to urinary estriol is particularly significant in diagnosis [2]. Biochemical studies have demonstrated that there are characteristic changes in the concentration of estrogens in both plasma and urine during the menstrual cycle. Most of these studies indicated that defined changes of urinary estrogens might be used as chemical indices to locate the start and finish of the probable fertile period [3]. Measurements of both plasma and urinary estrogens has proved to be equally useful in predicting fetal problems. However, whilst the urinary measurement of estrogens does usually indicate the amount secreted during the collection period, the concentration in plasma may be altered by rapid fluctuations in steroid level [4].

In clinical practice estrogens are usually determined using radioimmunoassay (RIA) methods [5, 6]. However, the increasing proliferation of RIA has raised serious problems associated with the handling of radiolabelled material and the waste disposal of radioactivity. Moreover, cross-reaction has made it evident that the estrogen levels measured in urine by RIA are considerably overestimated [7]. Therefore, chromatographic techniques have been extensively employed for measuring estrogens in body fluids of both pregnant and non-pregnant women as an alternative to RIA [1, 3, 8, 9]. Nevertheless, in classical liquid chromatography the separation is slow and not complete. As a result of these factors, the method is still not fully satisfactory with respect to speed, reliability and simplicity.

Recently, attempts have been made to utilize cyclodextrins in order to improve the high-performance liquid chromatographic separation and resolution of estrogenic steroids [9-11]. Cyclodextrins (CD) are a group of oligosaccharides that contribute to several guest-associated phenomena in solution including an increased aqueous solubility of certain compounds. The utilization of cyclodextrin inclusion processes in HPLC has been based on two different approaches. The first relies on the use of cyclodextrins chemically bonded to silica phases. In the second approach cyclodextrins are applied as mobile phase components, utilizing their selective complexation properties in a reversed-phase system [12].

The present work was aimed at the investigation of, firstly, the HPLC separation of estriol, estradiol and estrone using β-cyclodextrin as a mobile phase modifi-
er, and then the development of a reliable and simple HPLC assay for quantifying estrogens in the urine of pregnant and non-pregnant women.

Experimental

Reagents

Estrogens (estriol, estradiol and estrone) were purchased from Aldrich Europe: β-cyclodextrin was supplied by Chinoin (Budapest, Hungary). The solvents used as mobile phases in HPLC were purified by double distillation and filtering through a 1.5 μm membrane. The β-CD was added to mobile phase (25:75, v/v, acetonitrile in water) to give a final concentration of 2 mM, 4 mM, 6 mM, 8 mM, 10 mM, 12 mM, 14 mM and 16 mM.

Stock solutions of standards were prepared in methanol at a concentration of 1 mg/ml. From this stock solution, appropriate injection standard solutions were prepared before first evaporating the required volume of the stock solution in a vacuum and adding the chromatographic mobile phase. Separate standards were used at each concentration for the calibration curves.

Samples

Urine samples from pregnant subjects were taken between the 18th and 38th week of pregnancy. The urine was collected daily, and the total amounts were measured. From the daily urine sample, (average 1.2 l) a portion of 50 ml was taken for analysis. Overnight urine samples were collected from non-pregnant subjects. Overnight urine samples, defined as the first urine passed after rising from bed in the morning, were collected daily through the menstrual cycle. From the total amount of overnight urine (av. 300 ml) a 25 ml sample was taken for analysis.

Sample preparation

To determine total estrogens a 50 ml portion of urine (pregnant subjects) was mixed with 7 ml of concentrated hydrochloric acid and heated at 90 °C for 1 h. After hydrolysis, 30 μg (30 μl of a methanolic solution 1000 μg/ml) of 4-phenylphenol (internal standard) was added to the sample. Then the mixture was extracted three times with 10 ml portions of diethyl ether. The organic phases were combined and washed twice with 20 ml portions of NaHCO3/NaOH buffer (pH 10.5), and then with 20 ml portion of water. The organic phase was dried by the addition of 5 g anhydrous sodium sulphate. After filtration the sample was evaporated under reduced pressure almost to dryness. The residue was redissolved in 1 ml of mobile phase. A 20 μl portion of the solution was injected into the chromatograph.

The volume of urine sample from non-pregnant subjects was 25 ml. Therefore, with the exception of the internal standard half quantities of the reagents were used. Due to the relatively low fluorescence intensity of 4-phenylphenol, 100 μg of internal standard was added.

High-Performance Liquid Chromatography

Urine extracts from pregnant women have been analyzed with UV detection using the equipment described below: The liquid chromatograph consisted of an analytical solvent pump (Knauer's A0307), UV/VIS photometer (Knauer's A0293), column box and a linear recorder (Knauer). A Rheodyne Model 7125 injection valve was used for sample introduction. A 250 × 4.5 mm i.d. ODS column (from Beckman) was used at 40 °C. The UV detector was operated at 280 nm. The mobile phase consisted of water-acetonitrile (25:75, v/v); flow-rate 1 ml/min; column temperature 40 °C. The β-cyclodextrin was added to the mobile phase to a final concentration of 14 mM.

As the levels of estrogens in late-pregnancy urine are about 1000 times higher than those in non-pregnancy urine the following equipment was applied to the latter samples; The liquid chromatograph, consisting of an analytical solvent pump, Kontron SFM 25 spectrofluorimetric detector, and a linear recorder, was a product of Kontron. A Rheodyne Model 7125 injection valve was used for sample introduction. A 250 × 4.5 mm i.d. ODS 5 μm column (from Kontron) was used. The fluorescence detector was operated at λexe = 280 nm and λem = 312 nm. The mobile phase consisted of an acetonitrile-water system (25:75, v/v); flow rate 2 ml/min; column temperature 20 °C. The β-cyclodextrin was added to the mobile phase to a final concentration of 14 mM.

Result and Discussion

Chromatographic Conditions

The influence of mobile phase composition (without cyclodextrin) on the apparent capacity factors (k') is exemplified in Figure 1 by the behaviour of estriol estradiol and estrone. As can be seen, estradiol and estrone are practically not separated and the separation between estradiol and estriol is better at lower concentrations of acetonitrile in the mobile phase. The optimal mobile phase composition lies between 25 to 40 % (v/v) of acetonitrile in water. However, due to the poor solubility of β-cyclodextrin in water, a mobile phase with 25 % (v/v) of acetonitrile was chosen for further investigation.

Figure 2 shows the influence of β-CD concentration (mM) on the apparent capacity factors (k'). The mobile phase used was acetonitrile-water (25:75, v/v). The separation between estrone and estradiol was greatly improved in comparison to the separation obtained with a mobile phase without the addition of β-CD (see Figure 1).