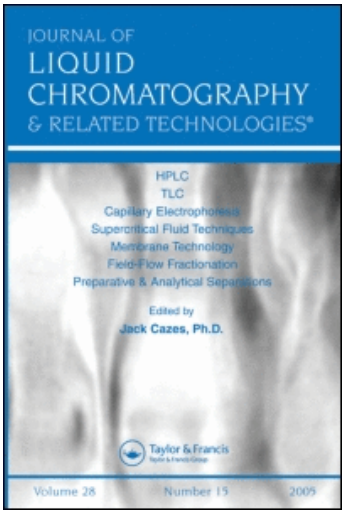

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IMPROVED SEPARATION OF C_{21}-STEROIDS OF WIDE POLARITY RANGE BY APPLICATION OF SEPHADEX LH-20, SUCCESSIVE SOLVENT SYSTEMS AND THIN-LAYER REFLECTANCE SPECTROMETRY

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ABSTRACT

A method is described for the separation on Sephadex LH-20 of C_{21}-steroids of wide polarity range using a mixture of dichloromethane, n-hexane and methanol. The relative amount of methanol is increased from 2%-5% during the chromatography. Steroids in the collected fractions are subjected to thin-layer chromatography - reflectance spectrometry and measured at 240nm (without treatment) or at 580nm (after tetrazolium blue treatment).

INTRODUCTION

Sephadex LH-20 has been employed for over two decades to separate steroids (1-4). Together with thin-layer reflectance spectrometry, it has been

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dedicated to Professor Egon Diczfalusy on the occasion of his 70th birthday

applied to the separation of large numbers of 3-oxo-Δ4- and saturated C_{21}-steroids (5).

Solvent systems successfully employed for the separation of polar C_{21}-steroids were unable to achieve separation of less polar compounds. Thus such systems could not be used for the the polar corticosteroids (6).

In order to optimize the resolution of mixtures of C_{21}-steroids of quite different polarities in a much shorter time and using much reduced solvent amounts, the polarity of the solvent has been increased during chromatography by slowly increasing the amount of methanol in the solvent system. Needless to say, the presently described system is less expensive than a gradient high performance liquid chromatograph (HPLC) system. Its performance is adequate for its intended separation of steroids before their determination by radioimmunoassay.

MATERIALS AND METHODS

Chemicals - Sephadex LH-20 (bead-size 25-100μm) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. The steroids 16α-hydroxyprogesterone (16α-hydroxy-4-pregnene-3,20-dione), corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione), aldosterone (3,20-dioxo-11β,21-dihydroxy-4-pregnen-18-al), cortisone (17α, 21-dihydroxy-4-pregnene-3,11,20-trione), cortisol (11β,17α,21-trihydroxy-4-pregnene-3,20-dione), tetrahydrocortisone (3α,17α,21-trihydroxy-5β-pregnane-11,20-dione) and tetrahydrocortisol (3α,11β,17α,21- trihydroxy-5β-pregnan-20-one were obtained from Steraloids Inc., Wilton, N.H., U.S.A. All reagents and solvents were from Merck, Darmstadt, F.R.G.

Solvent Systems: S-1, dichloromethane, n-hexane, methanol 50:48:2; S-2 dichloromethane, n-hexane, methanol 50:47.5:2.5 and S-3, dichloromethane, n-hexane, methanol 50:45:5.

Columns: glass columns with Teflon stop-cocks (30cm long and 1.2 x 0.1cm i.d.) filled with a Sephadex slurry prepared by addition of about 8g. Sephadex LH-20 swollen overnight in solvent S-1. The prepared was allowed to settle by gravity and had a bed height of 19.8cm. Column height and flow rate changes with the solvent system employed.

Thin-Layer Chromatography (TLC): Ascending one-dimensional TLC was carried out on precoated silica-gel layers (Merck AG) under saturated conditions (10), using as solvent systems ethyl acetate (saturated with water), n-hexane, ethanol, glacial acetic acid (72:13.5:4.5:10) for the analysis of 16α-hydroxyprogesterone, corticos-

terone and aldosterone [mobilities, hRf: 49, 43 and 31, respectively]; chloroform, methanol, water 90:10:1 for the analysis cortisone, cortisol, tetrahydrocortisone and tetrahydrocortisol [mobilities, hRf: 41, 29, 24 and 17, respectively; front 16.4cm.].

Visualisation - the Δ4-3-oxo-steroids were visualised under UV light at 240nm. Tetrahydrocortisone and tetrahydrocortisol were detected after treatment with tetrazolium blue (0.5% solution in methanol).

Reflectance Spectrometry - A single-beam Zeiss spectrophotometer type KM-3, Carl Zeiss, Oberkochen, F.R.G.) was used; 3-oxo-Δ4- steroids were measured at 240nm using a slit of 0.5 x 10mm, whereas tetrahydro-E and tetrahydro-F were measured at 580nm after tetrazolium blue treatment using a slit setting of 0.04 x 10mm.

Analysis of Fractions - Equal aliquots of the fractions collected from the Sephadex column were spotted on to the starting line of the thin layer with intervals of 0.5cm between them; mixtures of standards were chromatographed simultaneously at 2.5cm of each lateral border. After chromatography, the plates were dried and subjected to reflectance spectrometry with or without tetrazolium blue treatment.

RESULTS AND DISCUSSION

A mixture of C_{21}-steroids consisting of 200μg 16α-hydroxyprogesterone, corticosterone, aldosterone and cortisone together with 300μg of each of cortisol, tetrahydrocortisol and tetrahydrocortisone was applied to the Sephadex column prepared as described above and successively eluted with 40ml S-1 (flow-rate : 38ml/h), 40ml of S-2 (38ml/h) and 170ml of S-3 7ml/h). The bed length of 19.8 cm remained constant during the elution with S-1 and S-2, but increased during elution with S-3 from 21.1 to 21.6cm and flow-rate decreased to 7ml/h. During chromatography, 2.5ml fractions were collected and equal aliquots of each were investigated by TLC as described under Materials and Methods.

The results obtained by TLC-reflectance spectrometry and presented in the Figure indicate a complete separation of 16α-hydroxyprogesterone (tubes 12-16), corticosterone (19-24), aldosterone (25-33), cortisone (37-44) and cortisol (50-62) as well as between the urinary corticosteroid metabolites tetrahydrocortisone (53-65) and terahydrocortisol (75-98); cortisol and tetrahydrocortisone remained unresolved.

The solvent systems S-1 (2% methanol) and S-3 (5% methanol) have been used before for the separation of C_{21}-steroids; the former is more suitable for the less polar steroids and the latter for the more polar ones (6). Although the system S-1 can be

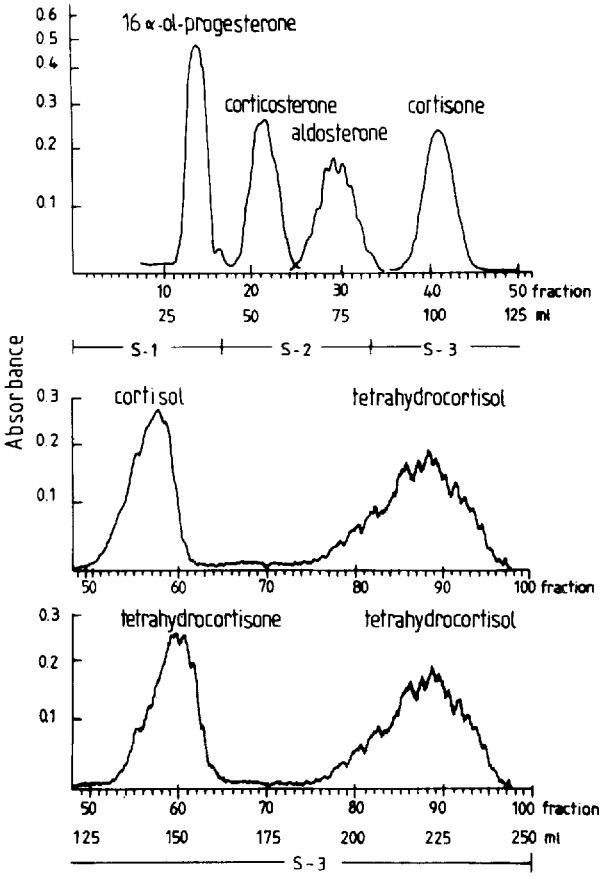

FIGURE Separation of a mixture of 16α-hydroxyprogesterone, corticosterone, aldosterone, cortisone, cortisol, tetrahydrocortisone and tetrahydrocortisol by gel column chromatography using a sequence of three solvent systems (dichloromethane n-hexane methanol: 50: 48: 2 (S-1), 50:47.5:2.5 (S-2), 50:45:5 (S-3). Elution profiles were obtained by thin-layer reflectance spectrometry.

employed for the separation of the polar compounds tetrahydrocortisone and tetrahydrocortisol, twice as much solvent is required (520ml instead of 250ml); on the other hand, solvent S-3 proved inadequate for the separation of less polar steroids. By increasing the amount of methanol in the system dichloromethane - n-hexane - methanol from 2% through 2.5% up to 5%, the mixtures could be investigated in a much shorter time with smaller solvent amounts. This increase must, however, be carried out gradually. Thus, a one-step increase from 2% to 5% gave rise to a sudden swelling of the Sephadex in the column. The amount of methanol in the solvent system is therefore a limiting factor and cannot be increased further. The application of the present method for the separation of five C_{21}-steroids - progesterone, 17α-hydroxy-progesterone, corticosterone, cortisone and cortisol from human plasma before their determination by radioimmunoassay will be published elsewhere (7).

Several solvent systems as effluents for Sephadex gel chromatography have been employed for the separation of mixtures of steroid conjugates (8) or in the group separation of steroid oestrogens (9) in free, glucosiduronate and sulphate (mono-, di- and trisulphate) fractions as well as corticosteroids (10). The present work indicates that with suitable choice of solvent systems and gradual increase of polarity, such a model can be useful for the separation of mixtures of free steroids of wide polarity range.

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