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APPLICATION OF ION-EXCHANGE AND LIPOPHILIC-GEL CHROMATOGRAPHY TO THE PURIFICATION AND GROUP FRACTIONATION OF STEROIDAL SPIROLACTONES, ISOLATED FROM BIOLOGICAL FLUIDS

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SUMMARY

Chromatography of steroidal spirolactones on DEAE-Sephadex A-25 under selected pH conditions allowed efficient separation of these compounds from other steroids and many of the endogenous components of human urine. The spirolactones were recovered in high yield, mostly over 90%. Lipophilic-gel chromatography provided a useful method for group fractionation of mixtures of these spirolactones with high recoveries (generally over 90%), unaffected by the presence of endogenous material from normal human urine.

INTRODUCTION

Over the past ten years an increase in the availability of stable isotopes coupled with improvements in gas chromatography–mass spectrometry (GC–MS) has provided new techniques which offer the required specificity and sensitivity for drug metabolism studies. However, the large amounts of endogenous material present in crude extracts of biological fluids may often limit the value of combining stable-isotope labelling with GC–MS. This is a particular problem when steroid drugs are being studied, as they have similar properties to endogenous steroids present in extracts of biological fluids from experimental animals and man. The accompanying materials in such extracts may co-chromatograph with the drug and its metabolites, interfering with the interpretation of the resulting mass spectral data, and sometimes leading to excessive contamination of the ion source with a consequent loss in sensitivity. Thus, to maximise the effectiveness of applying GC–MS and stable isotope labelling to studies of steroid drug metabolism, it is of value to purify and partially fractionate crude biological extracts prior to analysis.

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During a study of the metabolism of orally administered potassium canrenoate (SC-14266, I), successive chromatography of steroidal spirolactones on DEAE-Sephadex A-25 and on a lipophilic gel (Lipidex 5000) has been investigated as a potential general procedure for purifying and fractionating this class of steroid drugs, isolated from biological fluids. The results of this investigation suggest that these techniques offer a convenient way to purify and fractionate steroidal spirolactones, isolated from biological fluids, prior to GC–MS analysis.

MATERIALS AND METHODS

Analar-quality solvents, which were redistilled before use, and all other reagents were supplied by Hopkin and Williams, Chadwell Heath, Great Britain, unless otherwise stated. Ethanol was obtained as absolute alcohol from James Burrough, London, Great Britain; dichloromethane and tetrahydrofuran (THF) were obtained from BDH, Poole, Great Britain.

Reference compounds I and IIa (see Fig. 1) were supplied by G. D. Searle & Co., Morpeth, Great Britain, compounds III and IV by the Analytical Development Laboratory, G. D. Searle & Co., High Wycombe, Great Britain, and compounds IIb and IIc were obtained from Dr. A. Karim, Department of Drug Metabolism and Radiochemistry, Searle Labs., Skokie, Ill. (U.S.A.).

Synthesis of additional reference compounds

Va. 3β-Hydroxy-3-deoxocanrenone (3β,17β-dihydroxy-17α-pregna-4,6-diene-21-carboxylic acid γ-lactone). Canrenone (342 mg) and sodium borohydride (50 mg) were dissolved in ethanol (25 ml). The mixture was allowed to stand at room temperature and the reaction was monitored by thin-layer chromatography (TLC). After 5 h, the excess of sodium borohydride was destroyed by the addition of a few drops of acetic acid. The alcoholic solution was reduced in volume; water was added and the product was extracted with ether. After drying over anhydrous sodium sulphate, the ether was evaporated and the residue recrystallised from hot acetone–*n*-hexane to give a white solid (210 mg). The material was purified by lipophilic-gel chromatography on Lipidex 5000, m.p. 168–170° (found: C, 77.02; H, 8.86; $C_{22}H_{30}O_3$ requires: C, 77.15; H, 8.83). Elemental analysis was carried out by CHN Analysis, South Wigston, Great Britain. Mass spectral analysis by chemical ionisation with isobutane as the reagent gas showed ions at *m/e* 343 (MH^+, 96.6%) and *m/e* 325 ($MH^+ - 18$, 100%).

The assignment of the stereochemistry of the allylic alcohols derived from canrenone is tentative and was partly based on their oxidation with the enzyme cholesterol oxidase, whose substrate specificity for 3β-hydroxy-steroids has been well established by Smith and Brooks[1]. To a solution of the allylic alcohol (50 μg, 1.31 μmole) in isopropanol (100 μl) and phosphate buffer (3 ml) was added a solution of cholesterol oxidase from *Brevibacterium sterolicum* (50 μg, 20342 U/g, supplied by Dr. T. Uwajima[2]) in isopropanol (50 μl). The solution was allowed to stand overnight and was extracted with ethyl acetate (3 × 10 ml). The ethyl acetate layer was washed with water and dried over sodium sulphate. TLC and GLC analysis of the residue showed quantitative conversion to canrenone.

Vb. 3β,15α-Dihydroxy-3-deoxocanrenone (3β,15α,17β-trihydroxy-17α-pregna-4,6-diene-21-carboxylic acid γ-lactone). To 15α-hydroxycanrenone (1 mg) in ethanol

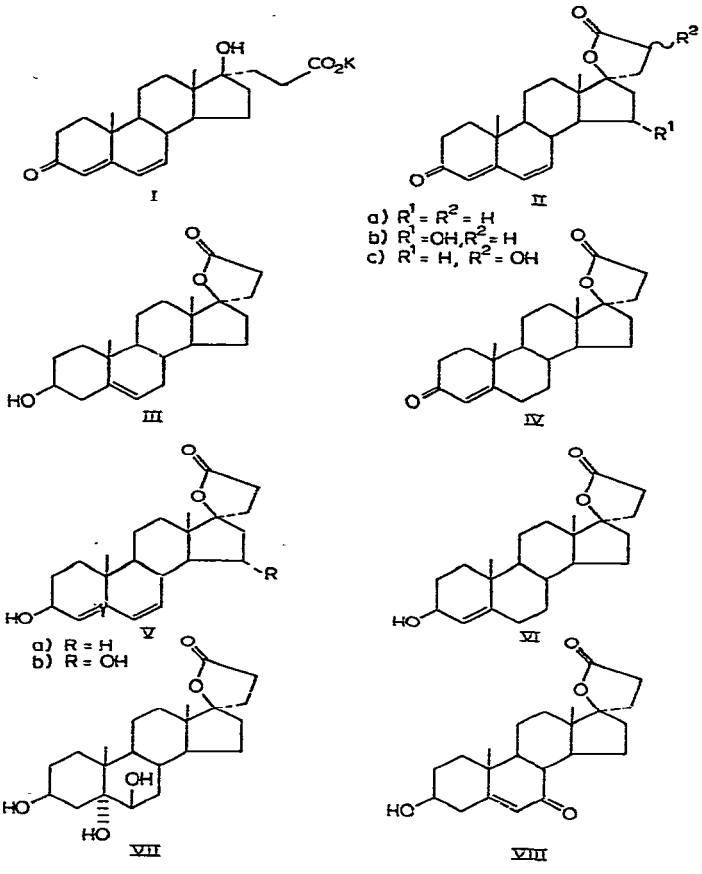

Fig. 1. Chemical structures of reference compounds. I = Potassium canrenoate (SC-14266); IIa = canrenone (17β-hydroxy-3-oxo-17α-pregna-4,6-diene-21-carboxylic acid γ-lactone); IIb = 15α-hydroxycanrenone (15α,17β-dihydroxy-3-oxo-17α-pregna-4,6-diene-21-carboxylic acid γ-lactone); IIc = 21ξ-hydroxycanrenone (17β,21ξ-dihydroxy-3-oxo-17α-pregna-4,6-diene-21-carboxylic acid γ-lactone); III = andrenolactone (3β,17β-dihydroxy-17α-pregn-5-ene-21-carboxylic acid γ-lactone); IV = 6,7-dihydrocanrenone (17β-hydroxy-3-oxo-17α-pregn-4-ene-21-carboxylic acid γ-lactone); Va = 3β-hydroxy-3-deoxocanrenone (3β,17β-dihydroxy-17α-pregna-4,6-diene-21-carboxylic acid γ-lactone); Vb = 3β,15α-dihydroxy-3-deoxocanrenone (3β,15α,17β-trihydroxy-17α-pregna-4,6-diene-21-carboxylic acid γ-lactone); VI = 3β-hydroxy-3-deoxo-6,7-dihydrocanrenone (3β,17β-dihydroxy-17α-pregn-4-ene-21-carboxylic acid γ-lactone); VII = 3β,5α,6β,17β-tetrahydroxy-17α-pregnane-21-carboxylic acid γ-lactone; VIII = 7-keto-andrenolactone 3β,17β-dihydroxy-7-oxo-17α-pregn-5-ene-21-carboxylic acid γ-lactone).

(0.75 ml) was added sodium borohydride in ethanol (50 μl, 10 mg/ml). The mixture was allowed to stand at room temperature and the reaction was monitored by TLC. After 6 h, the excess of reagent was destroyed with acetic acid, the alcohol volume reduced, water added, the product extracted with chloroform and purified by lipophilic-gel chromatography on Lipidex 5000. Mass spectral analysis by chemical ionisation with isobutane as reagent gas showed ions at *m/e* 359 (MH^+, 13.5%), *m/e* 341 ($MH^+ - 18$, 100%) and *m/e* 323, ($MH^+ - 36$, 86.9%).

VI. 3β-Hydroxy-3-deoxo-6,7-dihydrocanrenone (3β,17β-dihydroxy-17α-pregn-4-ene-21-carboxylic acid γ-lactone). Similarly, reduction of 6,7-dihydrocanrenone with sodium borohydride in methanol gave the corresponding allylic alcohol (m.p. 184–185°, methanol–water).

VII. 3β,5α,6β,17β-Tetrahydroxy-17α-pregnane-21-carboxylic acid γ-lactone. To a stirred solution of andrenolactone (10 mg, 0.02 mmole) in dry dichloromethane (3 ml) was added slowly a solution of *m*-chloroperbenzoic acid (10 mg, 0.05 mmole). The solution was stirred at room temperature for 1 h. TLC comparison with andrenolactone showed complete conversion of starting material to the epoxide. The reaction mixture was then treated with a solution of sodium sulphite to decompose the excess of *m*-chloroperbenzoic acid and then with a sodium bicarbonate solution. It was extracted with dichloromethane (2 × 50 ml). The usual work-up gave the crude epoxide, which was used directly for the following transformation.

To a solution of andrenolactone 5α,6α-epoxide (30 mg, 0.08 mmole) in acetone (1.5 ml) was added with stirring a solution of periodic acid ($HIO_4 \cdot 2H_2O$, 31 mg, 0.13 mmole) in water (0.5 ml). The solution was stirred for 1 h. TLC showed complete conversion of the starting material to the desired triol. The reaction mixture was diluted with water and extracted with chloroform (3 × 50 ml). The combined chloroform layer was washed with water and dried over anhydrous sodium sulphate. Removal of chloroform on a rotary evaporator gave a colourless solid (26 mg). It was recrystallised from aqueous acetone, m.p. 110°.

The tri-TMS ether of the triol was prepared by reaction of the triol (500 μg) in pyridine (100 μl) with a mixture of BSTFA–TSIM (3:1) at 100° for 1 h. The reaction mixture was then evaporated to dryness with a stream of nitrogen at 60° and the residue was dissolved in heptane (100 μl). This solution was passed through a column of Lipidex 5000 in heptane (4 cm, Pasteur pipette), 2-ml fractions were collected and analysed by GLC. The triol tri-TMS ether was found in the 4th and 5th fractions. GLC ($I^{OV-1}_{250°}$ 3344). The retention index corresponded to that expected for the tri-TMS ether.

VIII. 7-Keto-andrenolactone (3β,17β-dihydroxy-7-oxo-17α-pregn-5-ene-21-carboxylic acid γ-lactone).

(a) Andrenolactone acetate. To a solution of andrenolactone (34 mg) in dry pyridine (0.2 ml) was added acetic anhydride (1 ml); then the mixture was refluxed for 1 h. The solution was evaporated to dryness on a rotary evaporator, diluted with water and extracted with ether (3 × 25 ml). The combined ether layer, after washing with water, was dried over anhydrous sodium sulphate; the ether was then removed. TLC and GLC analysis of the residue (32 mg) showed complete conversion of andrenolactone to its acetate. The crude acetate was used as such without further purification for the oxidation.

(b) 7-Keto-andrenolactone acetate. The allylic oxidation of andrenolactone acetate was carried out according to Heusler and Wettstein's method[3] with modifications. Chromium trioxide (170 mg, 1.7 mmole) was dissolved in dry *tert.*-butanol (0.5 ml) with slight warming. To this red–orange solution was added successively carbon tetrachloride (0.5 ml), acetic acid (0.5 ml) and acetic anhydride (0.2 ml). The resultant clear dark-orange solution was used for the oxidation.

To a solution of crude andrenolactone acetate in carbon tetrachloride (1 ml) was slowly added the solution of *tert.*-butyl chromate reagent prepared as above. The reaction mixture was then refluxed for 8 h. GLC analysis of an aliquot showed 85%

conversion to the peak corresponding to 7-keto-andrenolactone acetate [$I_{250°}^{OV-1}$3390]. The solution was cooled and treated with methanol (1 ml) to destroy any excess *tert.*-butyl chromate. The solvents were removed on a rotary evaporator and the residue treated with saturated aqueous oxalic acid and extracted with ether (4 × 50 ml). 7-Keto-andrenolactone acetate thus obtained after the usual work-up was purified through a short column of Lipidex 5000 (6 cm, Pasteur pipette), using toluene as solvent. GC–MS analysis of the compound gave the expected ion at *m/e* 340 corresponding to loss of acetic acid from the molecular ion. A portion of 7-keto-andrenolactone acetate in THF was hydrolysed with saturated aqueous potassium carbonate at room temperature overnight. After the usual work-up it gave the free alcohol which was derivatised and analysed by GLC and GC–MS ($I_{250°}^{OV-1}$ 3311).

Thin-Layer Chromatography (TLC)

This was carried out on commercial precoated glass plates (silica gel 60 F_{254}, 20 × 20 cm, layer thickness 0.25 mm, No. 5715, E. Merck, Darmstadt, G.F.R.). Chromatograms were developed over a distance of 15 cm in glass tanks, lined with Whatman No. 1 filter paper. The atmosphere in the developing tanks was allowed to equilibrate for 2 h before use. Solvent systems for the development of chromatograms were: (1) chloroform–methanol (9:1, v/v); (2) ethyl acetate.

Compounds were detected by viewing under UV light (254 nm) and by spraying chromatograms with a mixture of water–ethanol–conc. sulphuric acid (18:1:1) by volume and heating for 7 min at 120°.

Gas chromatography (GC)

Samples were chromatographed using a Pye Model 104 gas chromatograph (Pye Unicam, Cambridge, Great Britain) fitted with a flame ionisation detector. Analysis was carried out in glass columns (1.5 m × 4 mm I.D., or 1 m × 2 mm I.D.) packed with Dexsil 300 GC (1 %) on 100–120 mesh Gas-Chrom Q (Phase Separations, Queensferry, Great Britain), or in 50-m long open tubular glass columns containing OV-1 as the stationary phase. Chromatography was performed at column and injection temperatures of 270 and 290° for the packed columns, and 250 and 270° for the open tubular columns. Nitrogen was used as the carrier gas at flow-rates of 50 ml/min in the packed columns and 2 to 5 ml/min in the open tubular columns.

Trimethylsilyl (TMS) derivatives were prepared by treating the samples with N,O-bis(trimethylsilyl)acetamide or N,O-bis-(trimethylsilyl)trifluoroacetamide in pyridine (100 μl, Pierce and Warriner, Chester, Great Britain). The mixtures were heated at 60° for 1 h, evaporated to dryness under a stream of nitrogen and the residues dissolved in a 100-μl ethanol solution of 17β-hydroxy-3-oxo-6,6-spirocyclopropyl-17α-pregna-1,4-diene-21-carboxylic acid γ-lactone (0.23 μg/μl, SC-25951) for GC. Calibration curves were constructed by mixing known amounts of the reference compounds or their TMS derivatives with this internal standard solution. The concentrations of the compounds in the column fractions were read from these calibration graphs, to determine the efficiency of recovery.

GC–MS analysis of single reference compounds was done on a capillary column under the following conditions. Instrument, LKB 9000; column, OV-1; length, 30 m; temperature, 270°; carrier gas, helium; flow-rate, 12 ml/min.

DEAE-Sephadex A-25 chromatography

Procedure 1: based on lactone cleavage with potassium hydroxide. DEAE-Sephadex A-25 (2 g, chloride form) was stirred at room temperature in 10% (w/v) ammonia solution (25 ml), filtered off, washed with water and then methanol; the excess solvent was removed under suction. The gel was then suspended in 50% aqueous methanol (20 ml) and poured into a glass chromatography tube (10 cm × 1.8 cm I.D.). A 50% aqueous methanolic solution (10 ml) of steroidal spirolactones (1 mg), potassium canrenoate (1 mg) in the presence or absence of other steroids, or a residue (50 to 200 mg) from a urine extract was carefully adjusted to pH 6 with aqueous acetic acid (10%, v/v) and passed through the column, recycling the eluate from urine extracts once through the column. The column was eluted with 50% aqueous methanol (150 ml) and the combined eluates were evaporated to dryness. The residue was dissolved in methanol (10 ml), and refluxed with 0.1 *N* potassium hydroxide solution (10 ml) for 1 h. The solution was adjusted to pH 8 with aqueous acetic acid (10%, v/v) and passed through a fresh column of DEAE-Sephadex A-25 (basic form, 2 g). Then the column was eluted with 50% aqueous methanol (150 ml) to recover the neutral steroids and/or neutral endogenous material, followed by elution of the column with 1% acetic acid in methanol (150 ml) to yield the purified steroidal spirolactones. The recovery of the spirolactones using this technique was assessed by GC, as described above. The purity of the spirolactones recovered from urine samples was assessed by TLC and GC.

Procedure 2: based on lactone cleavage with Triton B. In this milder procedure, designed to reduce the exposure of metabolites to strong base, the residue obtained from the combined eluates from the first stage was not dissolved in methanol. Instead, it was treated at room temperature with methanolic Triton B (benzyltrimethylammonium hydroxide, 0.1 ml of 40% (w/v) solution) for 15 min. This period of treatment was found most suitable to achieve opening of the spirolactone ring. The elution was effected as in Procedure 1.

In experiments designed to assess recoveries from the DEAE-Sephadex procedures alone, the residues obtained from the eluates were purified by dissolving in toluene and filtering through a short column (4 cm × 5 mm) of Lipidex 5000.

Lipophilic-gel chromatography

Hydroxyalkoxypropyl Sephadex, containing approximately 50% hydroxyalkoxy chains, was obtained as Lipidex 5000 from Packard Instruments, Caversham, Great Britain. Omnifit glass chromatography tubes and PTFE fittings were used throughout (Biolabs., Cambridge, Great Britain) and fractions were collected using a LKB 7000 Ultrorac fraction collector (LKB, South Croydon, Great Britain). Gravity flow columns (50 × 0.65 cm I.D., 12.8 ml) were prepared according to the manufacturer's instructions.

Reference compounds (50 to 200 μg) dissolved in toluene (100 or 150 μl) were applied to the columns, with the addition of ethanol (10 to 20 μl) when required for solution. The compounds were washed onto the gel with toluene (3 × 100 μl) and eluted with toluene at a flow-rate of 0.32 ml/min. Fractions (1 ml) were collected and assayed by TLC and GC. Reference compounds were also added to residues of control urine extracts and chromatographed in the same manner. Columns were cleaned after

chromatography of urine extracts by elution with toluene–isopropanol (3:1, v/v, 25 ml) and re-conditioned with three column volumes of toluene.

Extracts containing urinary metabolites of potassium canrenoate were dissolved in toluene (150 μl) and ethanol (10 μl), applied to the Lipidex 5000 column and washed onto the gel with toluene (3 × 100 μl). The compounds were eluted with toluene (100 ml) at 0.32 ml/min and then toluene–isopropanol (3:1, v/v, 25 ml), collecting 1-ml fractions. The isolated fractions were subjected to TLC and GC analysis as described above.

The standard elution volume (SEV) of each compound was calculated according to the method of Brooks and Keates[4]:

$$\text{SEV} = \frac{\text{measured elution volume of compound}}{\text{total column volume}} \cdot 100$$

Isolation and purification of potassium canrenoate metabolites present in human urine

Pre-treatment urine samples and samples containing potassium canrenoate metabolites were obtained from two healthy normal men, who received a single oral dose of potassium canrenoate (200 mg), containing an equimolar mixture of the drug and its 20,21-tetradeutero analogue. Aliquots of the pooled 0–24-h and pre-treatment urine samples were processed through Amberlite XAD-2 resin according to Bradlow's method[5]. The methanolic eluates were evaporated to dryness, the residues dissolved in water which was adjusted to pH 3 with dilute hydrochloric acid and extracted with chloroform to isolate the "free" fraction. Subsequently the conjugated fractions were hydrolysed with β-glucuronidase (Type H-2, 1000 Fishman units per ml of urine, Sigma, London, Great Britain) in acetate buffer (pH 5.0) at 37°. The released "aglycones" were isolated by chloroform extraction at pH 3.

Samples of the pre-treatment urine extract, containing added known amounts of reference spirolactones, were used to investigate the efficiency of the DEAE-Sephadex A-25 and lipophilic-gel chromatography of these compounds in the presence of endogenous material. Samples of the "free" and "aglycone" fractions of urine containing potassium canrenoate and its metabolites were analysed by lipophilic-gel chromatography on Lipidex 5000 before and after purification by DEAE-Sephadex chromatography, as described above. Selected metabolite fractions collected from the lipophilic gel were analysed by TLC and GC to compare the purity of similar metabolite fractions obtained with and without prior DEAE-Sephadex A-25 chromatography.

RESULTS

More than 90% of the endogenous material present in the "free" or "aglycone" fraction isolated from control human urine was eliminated by analysis on DEAE-Sephadex A-25 and in most cases more than 90% of added spirolactones were recovered from various mixtures of steroids and from urine extracts using this technique, as shown by the data in Table I. These results indicated that efficient recovery and purification of the spirolactones had been achieved. Further evidence of the very substantial elimination of endogenous material was provided by GC analysis of the trimethylsilylated residue from the final 1% acetic acid–methanol eluate, obtained

TABLE I

RECOVERY OF STEROIDAL SPIROLACTONES FROM MIXTURES OF STEROIDS OR NORMAL HUMAN URINE EXTRACTS FOLLOWING DEAE-SEPHADEX CHROMATOGRAPHY

Sample (mg)	*Spirolactone added to sample (mg)*	*Recovery of spirolactone (%)*
DEAE-Sephadex procedure 1		
Control urine extract (6)	Potassium canrenoate I (0.82)	95
Steroid mixture* (6.8)	Potassium canrenoate I (0.82)	93
	Canrenone IIa (0.050)	98
	Andrenolactone III (0.050)	99
	15α-Hydroxycanrenone IIb (0.050)	100
DEAE-Sephadex procedure 2		
	Canrenone IIa (0.5)	93
	Andrenolactone III (0.5)	90
	6-Hydroxy-6,7-dihydrocanrenone (0.1)	87
	Triol VII (0.1)	93
	Allylic alcohol Va (0.1)	91
	6,7-dihydroxy-6,7-dihydrocanrenone (0.1)	94
Control urine extract** (25)	Canrenone IIa (0.1)	78
	Andrenolactone III (0.1)	98
	6-Hydroxy-6,7-dihydrocanrenone (0.05)	45

* Mixture contained estrone, oestradiol, cortisone, tetrahydrocortisone, testosterone, etiocholanolone, 5α-androstane-5β,17β-diol, 3β-hydroxy-5-androsten-17-one and 3β-hydroxy-5α-androstan-17-one.

** Coprostanol was added as a reference compound to correlate retention times and to allow quantitative estimation.

from DEAE-Sephadex A-25 (Fig. 2B: sample injected was equivalent to 2.50 μg of original urine extract), in comparison with direct GC of 25 μg of (trimethylsilylated) urine extract (Fig. 2A).

Recovery of steroidal spirolactones from DEAE-Sephadex was also estimated using [1,2-$^3H_2$]-canrenone as shown in Table II. The apparent loss of 50% of radioactivity is attributed to enolisation of [1,2-$^3H_2$]-canrenone on base treatment. To circumvent this, the base-treated [1,2-$^3H_2$]-canrenone was worked up as usual and the [1-$^3H_2$]-canrenone thus obtained was passed through the DEAE-Sephadex procedure. Once again, recovery of the spirolactone was around 90% with negligible loss of the total radioactivity. Likewise, [1,2-$^3H_2$]-3β-hydroxy-3-deoxocanrenone, prepared from [1,2-$^3H_2$]-canrenone by sodium borohydride reduction, showed 90% recovery.

The lipophilic-gel chromatography of the reference spirolactones on Lipidex 5000 demonstrated the ability of this technique to separate these steroids into groups, as shown in Fig. 3. This group separation appeared to be controlled by the number of keto- and/or hydroxy-groups in the steroid molecule. The recovery and SEV of the reference compounds were unaffected by the presence of extracts of control urine and there was some resolution of the spirolactones from the endogenous components present in urine. Repeated use of the same Lipidex 5000 columns over eight months had little effect on the SEV values or recoveries of the spirolactones, as shown by the results in Table III.

The thin-layer chromatograms in Fig. 4 show some of the results of lipophilic-

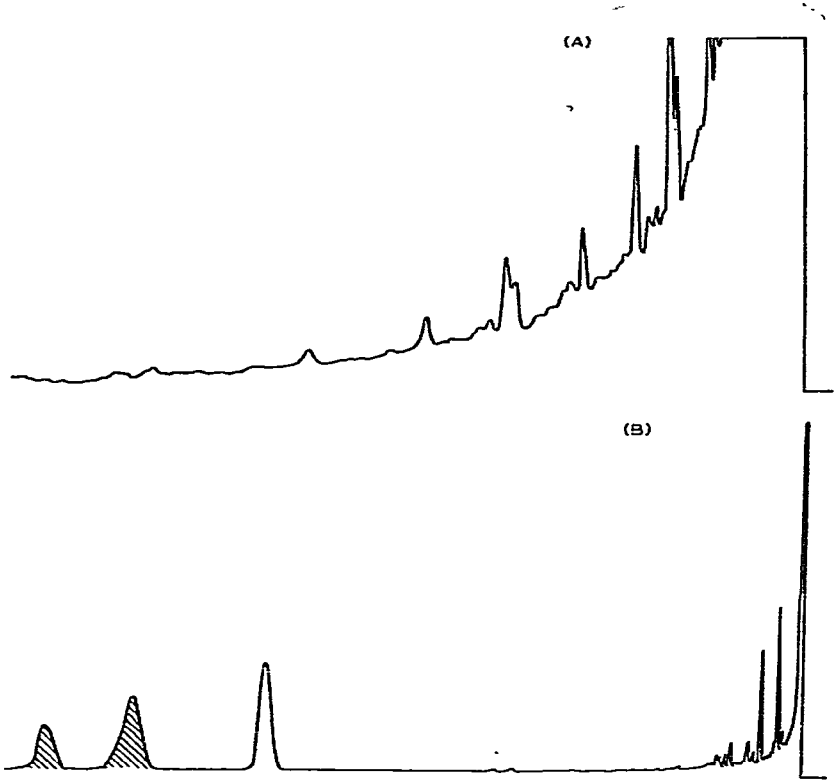

Fig. 2. Capillary gas chromatograms. (A) Free fraction of normal human urine obtained by extraction with chloroform at pH 3; (B) identical fraction of normal human urine, containing added spirolactones, after purification by DEAE-Sephadex A-25 Procedure I. Hatched areas correspond to reference spirolactones added to urine extract.

TABLE II

RECOVERY OF THE STEROIDAL SPIROLACTONES [1,2-$^3H_2$]-CANRENONE AND [1,2-$^3H_2$]-3β-HYDROXY-3-DEOXOCANRENONE FROM DEAE-SEPHADEX

Sample (μg)	*Initial counts* (dpm)*	*Counts of eluates obtained from DEAE-Sephadex*			
		Methanol eluate		*Methanol–acetic acid eluate*	
		dpm	*Initial count (%)*	*dpm*	*Initial count (%)*
DEAE-Sephadex procedure 1					
[1,2-$^3H_2$]-Canrenone (1000)	779913	454	0.06	348130	45
[1-$^3H_2$]-Canrenone** (4)	1881	33	1.8	1717	91.3
DEAE-Sephadex procedure 2					
[1,2-$^3H_2$]-Canrenone (50) + dosed urine extract (5000)	47140	209	0.44	21185	45
[1,2-$^3H_2$]-3β-Hydroxy-3-deoxocanrenone*** (60)	62576	7974	13	53280	85

* Philips P. W. 4510 scintillation analyser; samples were dissolved in toluene (10 ml) containing 2,5-diphenyl-oxazole (5 g/l) and 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)-benzene (0.25 g/l).

** Sample was obtained by treating [1,2-$^3H_2$]-canrenone with 0.1 *N* potassium hydroxide as before and by usual work-up.

*** Sample was obtained by sodium borohydride reduction of [1,2-$^3H_2$]-canrenone.

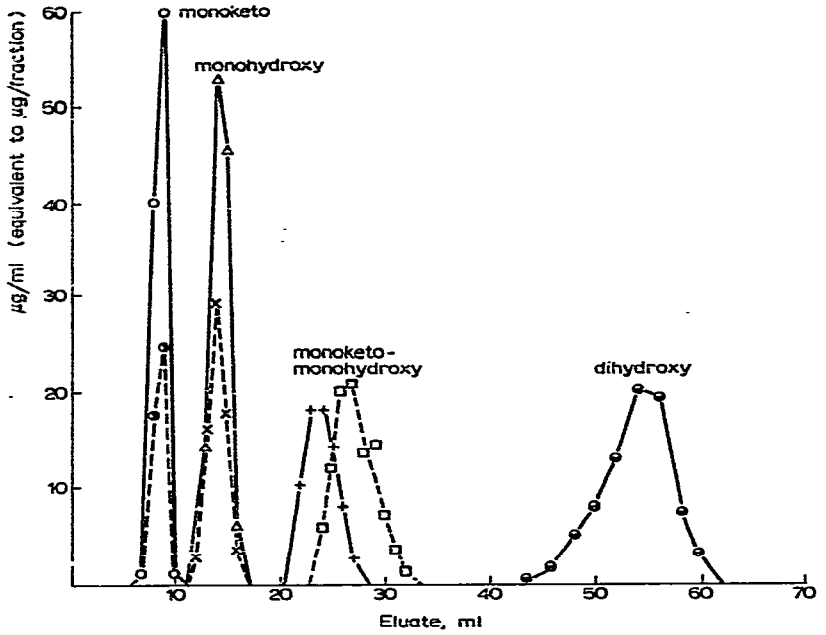

Fig. 3. Chromatography of reference spirolactones on Lipidex 5000 with toluene as the eluant at a flow-rate of 0.32 ml/min. ⊙ = canrenone (IIa); ◑ = 6,6-dihydrocanrenone (IV); △ = andrenolactone; × = $\Delta^{4,6}$-3β-hydroxy-3-deoxocanrenone (Va); ⊡ = 15α-hydroxycanrenone (IIb); + = 21-ξ-hydroxycanrenone (IIc); ◒ = $\Delta^{4,6}$-3β,15α-dihydroxy-3-deoxocanrenone (Vb).

gel chromatography of "free" and "aglycone" fractions obtained from the urine of men who had taken a single oral dose of potassium canrenoate (200 mg). Lipophilic-gel chromatography alone on Lipidex 5000 provided a fractionation of the material isolated from urine and revealed the presence of a number of previously unreported

TABLE III

DATA FOR ELUTION OF REFERENCE COMPOUNDS FROM LIPIDEX 5000 BY A TOLUENE SYSTEM

Reference compound	*Approximate SEV*			*Recovery (%)*		
	*Average**	*Range*	*Value for column after 8 months use*	*Average**	*Range*	*Value for column after 8 months use*
Canrenone (IIa)	66.7 (6)	64.5–70.3	66.4	104.0 (6)	92.0–113.7	105.4
6,7-Dihydrocanrenone (IV)	66.4	—	—	—	—	—
Andrenolactone (III)	112.5 (5)	105.5–117.2	105.5	116.0 (5)	110.6–126.7	97.9
3β-Hydroxy-3-deoxo-canrenone (Va)	107.4 (2)	105.5–109.4	—	—	—	—
15α-Hydroxycanrenone (IIb)	201.2 (6)	184.0–214.8	180.0	100.1 (5)	88.6–114.0	84.3
21ξ-Hydroxycanrenone (IIc)	183.6	—	—	—	—	—
3β,15α-Dihydroxy-3-deoxo-canrenone (Vb)	418.8 (2)	415.6–421.9	—	—	—	—

* Figures in parentheses denote the number of experiments from which the average values were calculated. The experiments were carried out during the initial 6 weeks following column preparation.

metabolites of potassium canrenoate in the urine extracts. However, metabolite fractions obtained from Lipidex 5000 after pre-treatment of the urine extracts with DEAE-Sephadex contained very little endogenous material, in contrast to the same fractions obtained using lipophilic-gel chromatography alone. For example, TLC analysis of the polar fraction of the "free" urine extract eluted from Lipidex 5000 by toluene–isopropanol (3:1, v/v) revealed two canrenoate metabolites at R_F 0.08 and 0.13 in the DEAE-Sephadex treated sample (Fig. 4). These two metabolites could not be detected in the corresponding fraction obtained without DEAE-Sephadex chromatography due to the large amount of endogenous material eluted in this fraction.

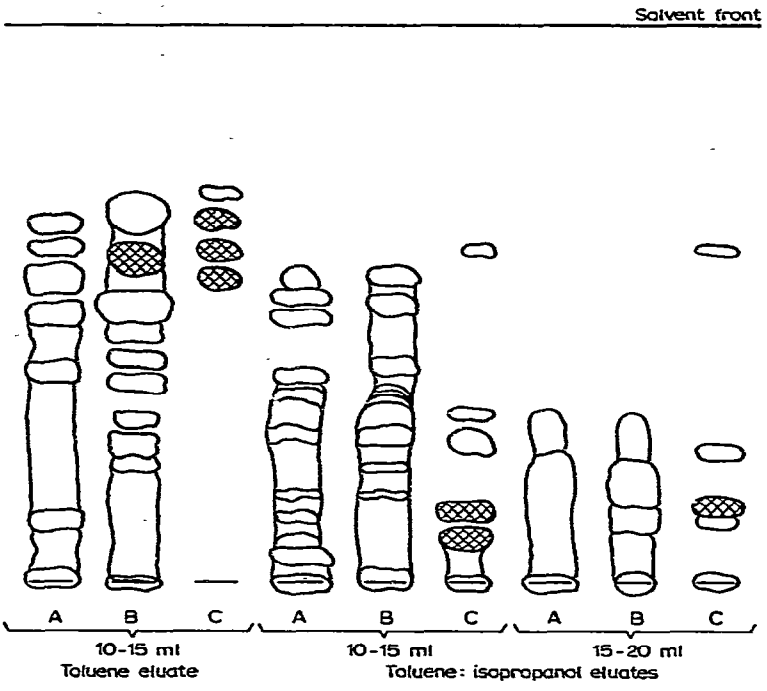

Fig. 4. Thin-layer chromatograms of selected fractions from Lipidex-gel chromatography. The chromatograms compare fractions from (A) control urine extract (B) extract from urine containing potassium canrenoate metabolites (C) extract of urine containing potassium canrenoate metabolites, purified by DEAE-Sephadex A-25 Procedure I, prior to Lipidex chromatography. Hatched areas correspond to suspected metabolites of potassium canrenoate.

DISCUSSION

The efficient recovery and purification of potassium canrenoate and related compounds from steroid mixtures and urine extracts obtained by the DEAE-Sephadex technique described above reflects an unique chemical property of this class of steroidal aldosterone antagonists. These properties are related to the presence of the 17α-propionic acid side chain which is characteristic of these compounds, and may be readily closed to the corresponding lactone under appropiate conditions. At pH 6 the 17α-propionic acid side chain of these compounds lactonised with the 17β-hydroxy

group. The resulting lactones passed through the DEAE-Sephadex column with the neutral steroids or neutral and basic endogenous materials, while acidic components present were retained by the basic DEAE-Sephadex. However, following hydrolysis of the lactone ring, chromatography at pH 8 led to quantitative retention of the anionic form of these spirolactones of the gel, whereas the majority of the neutral and basic components in the urine were eluted in the aqueous methanol. Subsequent elution of the gel with 1% acetic acid in methanol caused lactonisation of the 17α-propionic acid side chain with a consequent release of the spirolactones from the DEAE-Sephadex.

Since the purification of steroidal spirolactones in urine extracts obtained with DEAE-Sephadex is dependent on the presence and properties of the 17α-propionic acid side chain and 17β-hydroxy group, the method would appear to be generally applicable to any compounds in this class of steroids. One disadvantage of the method described was the risk of degradation of alkali-unstable metabolites when the spirolactone ring was hydrolysed with potassium hydroxide at elevated temperatures. However, the effective use of Triton B in methanol as a phase-transfer catalyst for hydrolysis at room temperature of the spirolactone ring of canrenone (IIa) and several related compounds, appears to offer a rapid mild method, less likely to degrade labile metabolites present in urine extracts.

The gel chromatography of the reference spirolactones on Lipidex 5000 in the presence or absence of extracts of control human urine appeared to be an effective method to separate these compounds into groups, and to separate them partially from some of the normal urinary constituents. The group separation depended predominantly on the numbers of keto and/or hydroxy groups in the compounds, as shown by co-elution of canrenone (IIa) and 6,7-dihydrocanrenone (IV), and the effective separation of these two compounds from andrenolactone (III) and 3β-hydroxy-3-deoxocanrenone (Va), which in turn were separated from 15α-hydroxycanrenone (IIb) and 3β,15α-dihydroxy-3-deoxocanrenone (Vb). The use of lipophilic-gel chromatography on hydroxyalkoxypropyl Sephadex to fractionate steroids into groups has been reported previously by Brooks and Keates[4] and by Anderson *et al.*[6] for naturally occurring steroids in man. These workers found a similar dependence of the separation on the number of keto and/or hydroxy groups in the steroids.

The results obtained in the present study provide good evidence that lipophilic-gel chromatography on Lipidex 5000 can be used to bring about group resolution of urinary metabolites of steroids other than those occurring naturally. This group resolution provided cleaner metabolite fractions suitable for GC and GC–MS analysis. In addition, SEV of metabolite fractions may give information about the type of metabolites present in the fraction by comparison with the SEV of reference compounds. The utility of the lipophilic-gel chromatography was confirmed by the effective group fractionation and partial purification of the urinary metabolites of potassium canrenoate. Some initial results of GC–MS analysis of these metabolite fractions has been recently reported[7], and confirms the presence of a number of previously unknown metabolites.

Lipophilic-gel chromatography provided some purification of the urinary metabolites. The combination of DEAE-Sephadex and lipophilic-gel chromatography provided very efficient purification of the spirolactone metabolites combined with an effective group separation, apparently based on the number of keto and/or hydroxy

groups in the metabolites. In the present study there was some evidence that some stage in the DEAE-Sephadex procedure, possibly treatment with potassium hydroxide at 65°, caused degradation of labile canrenoate metabolites. However, these labile metabolites were also found to be degraded by TLC and GC analysis of fractions obtained from Lipidex 5000 without initial chromatography on DEAE-Sephadex.

Thus, chromatography on DEAE-Sephadex A-25 provides a potentially general method for the concentration and purification of metabolites of steroidal spirolactones isolated from biological fluids. When this technique is combined with lipophilic-gel chromatography of the purified extracts on Lipidex 5000, it constitutes a novel general procedure for the concentration, purification and subsequent fractionation of metabolites of these steroid drugs. This combination of chromatographic methods provides metabolite fractions of adequate purity for GC–MS analysis. The low levels of endogenous materials present in metabolite fractions should allow more effective detection and identification of the metabolites. The previously unreported urinary metabolites of potassium canrenoate detected in the present study are currently under investigation by GC–MS in an attempt to elucidate their structures. The results of this work will be reported elsewhere.

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