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# DETERMINATION OF 17-OXOSTEROID GLUCURONIDES AND SULPHATES IN URINE BY LIQUID CHROMATOGRAPHY USING 2,4-DINITROPHENYLHYDRAZINE AS A PRELABELLING REAGENT FOR SPECTROPHOTOMETRIC DETECTION

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## SUMMARY

A direct, convenient method using high-performance liquid chromatography with spectrophotometric detection has been developed for the determination of conjugated 17-oxosteroids in urine without hydrolysis. Conjugated 17-oxosteroids are extracted with a Sep-Pak $C_{18}$ cartridge, prelabelled with 2,4-dinitrophenylhydrazine in trichloroacetic acid–benzene solution and then separated by high-performance liquid chromatography on a reversed-phase $C_{18}$ column using a mobile phase of 70% methanol in a buffer consisting of 50 m$M$ sodium acetate in 2% (v/v) acetic acid. The eluate is monitored by a spectrophotometer at 380 nm and a linear response was found for absorbance readings (peak heights) from amounts of various conjugated oxosteroids between 25 and 250 ng. The method provides a sensitive, reliable technique for the analysis of urinary 17-oxosteroid conjugates.

## INTRODUCTION

The measurement of urinary 17-oxosteroids, especially when fractionated chromatographically, has been useful in assessing androgen metabolism [1,2]. Numerous methods have been reported for urinary profiles of 17-oxosteroids including alumina column chromatography [3,4], gas chromatography (GC) [5–7] and gas chromatography–mass spectrometry [8]. In these methods, the conjugated steroids are determined indirectly following hydrolysis and solvolysis which suffer from incomplete deconjugation and artefact formation [9,10]. Also, site of conjugation is not determined by these methods which simply divide the 17-oxosteroid conjugates into glucuronide and sulphate fractions [11].

Recently, conjugated 17-oxosteroids have been assayed directly by reversed-phase high-performance liquid chromatography (HPLC) without hydrolysis [12]. Dansyl hydrazine was used to prelabel the conjugates for fluorescence detection.

In another method using reversed-phase HPLC, *p*-nitrophenylhydrazine was used to prelabel the conjugates for electrochemical detection [13].

In this paper we describe a sensitive direct HPLC method with spectrophotometric detection for the determination of urinary 17-oxosteroid conjugates using 2,4-dinitrophenylhydrazine as a prelabelling reagent.

EXPERIMENTAL

*Materials*

Androsterone glucuronide (AN-G) and etiocholanolone glucuronide (ETIO-G) were gifts from the steroid reference collection of the Medical Research Council (London, U.K.). Androsterone sulphate (AN-S), etiocholanolone sulphate (ETIO-S) and dehydroepiandrosterone sulphate (DHEA-S) were purchased from Steraloids (Wilton, NH, U.S.A.). All purchased steroid conjugates were sodium salts. Sep-Pak $C_{18}$ cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.). Trichloroacetic acid and 2,4-dinitrophenylhydrazine were from BDH (Poole, U.K.).

*Instruments*

The apparatus used consisted of an ETP/Kortec K35M high-performance liquid chromatograph (ETP/Kortec, Ermington, Australia) with an ETP/Kortec K95 variable-wavelength detector set at 380 nm for the 2,4-dinitrophenylhydrazine chromophore. HPLC was performed using a 150 mm × 3 mm I.D. stainless-steel column packed with 5 $\mu$m Spherisorb ODS 2 (Phase Separations, Clwyd, U.K.) using a slurry-packing apparatus (Alltech, Deerfield, IL, U.S.A.). Isocratic chromatography was performed with a mobile phase of 70% methanol in buffer at a flow-rate of 0.8 ml/min. The buffer consisted of 50 m$M$ sodium acetate in 2% (v/v) acetic acid. Water bath was set at 60°C for derivatisation.

*Reagent solutions*

2,4-Dinitrophenylhydrazine solution was prepared by dissolving 250 mg in 5 ml ethyl acetate. The reagent was freshly prepared prior to use. Trichloroacetic acid solution (TCA) was prepared at a concentration of 3.0 mg/ml in benzene. Steroid stock solutions AN-G, ETIO-G, AN-S, ETIO-S and DHEA-S were all dissolved in methanol at 1 mg/ml. For the steroid working solution, 100 $\mu$l of the steroid stock solution were dissolved in 10 ml methanol. This gives 200 ng per 20 $\mu$l which were evaporated to dryness under dry nitrogen at 40°C prior to derivatization.

*Extraction method*

A 1-ml urine sample was applied onto a Sep-Pak $C_{18}$ cartridge and washed with 5 ml of water. Conjugated 17-oxosteroids were eluted with 3 ml of methanol. One fifth of the eluate (0.6 ml) was evaporated to dryness under dry nitrogen at 40°C prior to derivatization.

*Labelling reaction*

To the standard or sample residue were added 10 $\mu$l of the 2,4-dinitrophenyl-hydrazine solution and 100 $\mu$l of the TCA. The solution was heated at 60°C for
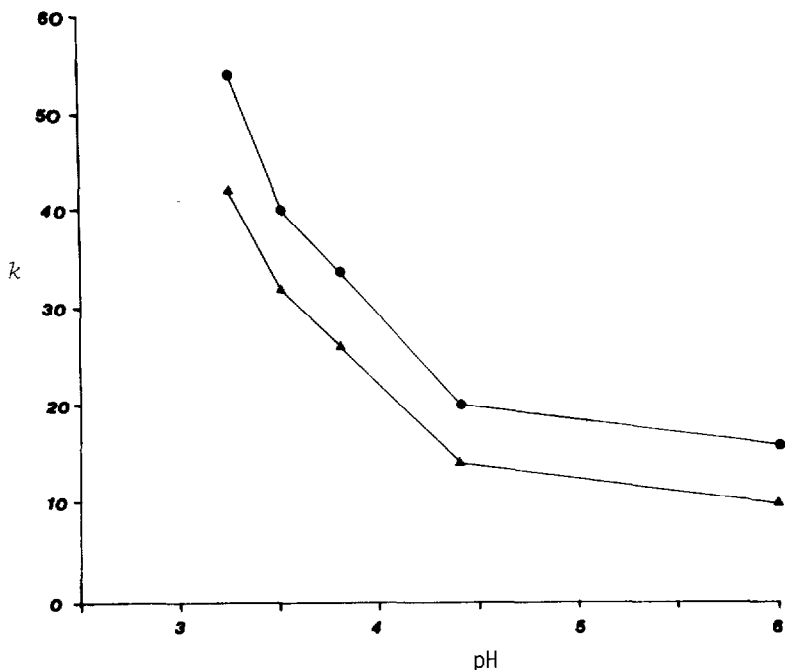
Fig. 1. Effect of pH on capacity factor ($k$). Mobile phase was 70% methanol in water with variation of pH from 3.5 to 6. (●) AN-G; (▲) ETIO-G.

20 min and then evaporated under dry nitrogen at 40°C and the residue dissolved in 25 $\mu$l of mobile phase. A 20-$\mu$l aliquot was analysed by HPLC.

RESULTS

*Dinitrophenylhydrazone absorption spectrum*

Dinitrophenylhydrazone of DHEA-S was prepared by reaction of DHEA-S with 2,4-dinitrophenylhydrazine in TCA–benzene solution. The absorption spectrum has a maximum at 380 nm and is stable in solution for two days at 4°C. The molar absorptivity ($\epsilon$) at this wavelength was 21 000.

*Effect of mobile phase composition*

To obtain good separation of 17-oxosteroid conjugates, solvent systems were investigated that took advantage of the ionizable properties of the conjugates. A decrease from pH 6 to 3.5 of the water used in 70% methanol solvent system gave increased retention on the $C_{18}$ column for the glucuronides with good separation from excess dinitrophenylhydrazine (Fig. 1).

By using 70% methanol in a buffer containing 6% acetic acid and varying concentrations of sodium acetate to achieve the same pH change as Fig. 1, glucuronides and sulphates showed opposite retention on the $C_{18}$ column (Fig. 2). With increasing concentration of sodium acetate the pH was increased and so the glucuronide capacity factors decreased. However, the capacity factors of the sul-
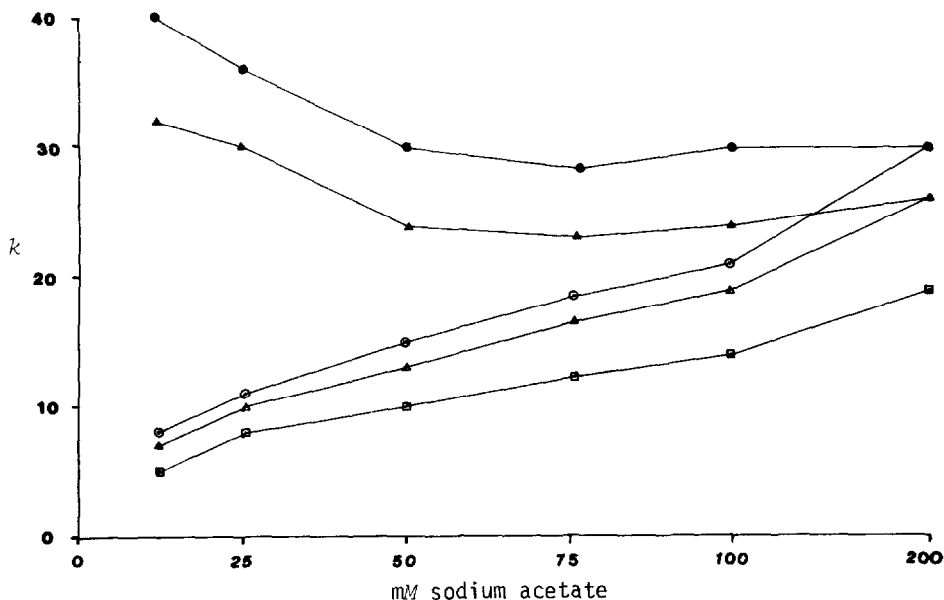
Fig. 2. Effect of sodium acetate concentration on capacity factor ($k$). Mobile phase was 70% methanol in buffer containing 6% acetic acid and sodium acetate. (●) AN-G; (▲) ETIO-G; (o) AN-S; (△) ETIO-S; (□) DHEA-S.

phates increased with increasing concentration of sodium acetate.

When 70% methanol in a buffer containing 2% acetic acid and varying concentrations of sodium acetate was used, the sulphates exhibited increased retention and thus a better separation from excess 2,4-dinitrophenylhydrazine was obtained. Using this system, the glucuronides were less retained which shortened assay time without loss of resolution.

The effect of methanol concentration on the capacity factors was investigated using a buffer containing 2% acetic acid and 50 m$M$ sodium acetate (Fig. 3). The optimum mobile phase composition was found to be 70% methanol in buffer containing 50 m$M$ sodium acetate and 2% acetic acid since it gave the best resolution within the shortest time. A chromatogram of a standard 17-oxosteroid conjugate mixture containing DHEA-S, ETIO-S, AN-S, ETIO-G and AN-G is shown in Fig. 4.

*Standard curve and sensitivity*

When absorbance at 380 nm (peak height) was plotted against injected amounts of 17-oxosteroid conjugate standards, a linear response was obtained between 25 and 250 ng. The detection limit for standards was about 2 ng with a signal-to-noise ratio of 2. When 1 ml of urine was used for the assay, the detection limit was ca. 1 $\mu$g per 100 ml.

*Precision and recovery*

Recovery experiments were carried out by adding 300 ng each of the five conjugated 17-oxosteroids to a 1-ml urine sample. As shown in Table I, conjugated
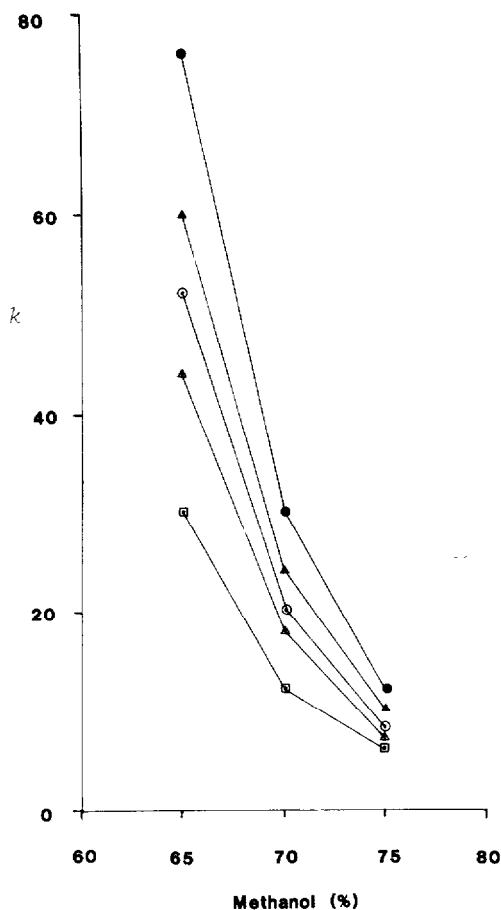
Fig. 3. Effect of methanol concentration on capacity factor ($k$). Mobile phase was different concentrations of methanol in buffer containing 2% acetic acid and 50 m$M$ sodium acetate. (●) AN-G; (▲) ETIO-G; (○) AN-S; (△) ETIO-S; (□) DHEA-S.

17-oxosteroids were recovered in the range 96–104% with a coefficient of variation (C.V.) in the range 2–4%.

*Typical chromatograms from urine samples*

Typical chromatograms obtained from normal adult male and female urine are shown in Fig. 5. All five conjugated 17-oxosteroids are clearly separated and peak identities attributed on the basis of comparison with authentic samples.

Chromatograms of 17-oxosteroid conjugates in urines from a prepubertal boy both pre and post human chorionic gonadotrophin (HCG) stimulation are shown in Fig. 6. Following HCG stimulation, there is a marked increase in the androsterone and etiocholanolone conjugates which improves their analysis when determining the ratio of etiocholanolone glucuronide to androsterone glucuronide in screening for 5$\alpha$-reductase deficiency.
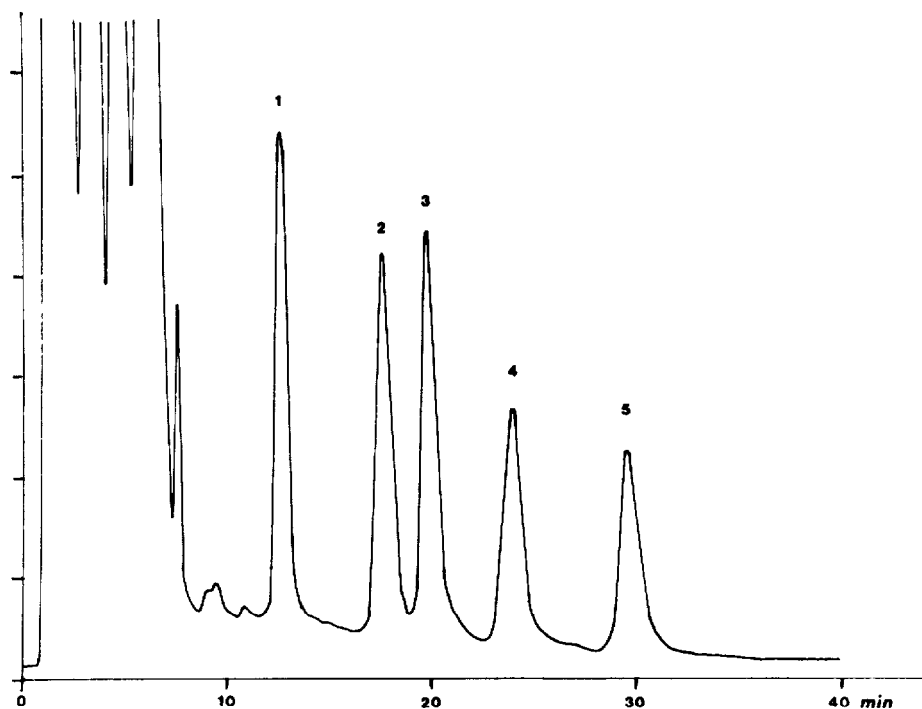
Fig. 4. Chromatogram of 2,4-dinitrophenylhydrazones of a conjugated 17-oxosteroid standard mixture. Mobile phase was 70% methanol in buffer containing 2% acetic acid and 50 m$M$ sodium acetate, flow-rate, 0.8 ml/min; spectrophotometric detection at 380 nm. Peaks: 1 = DHEA-S; 2 = ETIO-S; 3 = AN-S; 4 = ETIO-G; 5 = AN-G.

DISCUSSION

Urinary 17-oxosteroid conjugates reflect adrenal and testicular/ovarian C$_{19}$ steroid production as well as peripheral metabolism by 5$\alpha$-reductase. Most of the methods for 17-oxosteroid conjugate determination are indirect and require hydrolysis [3–8]. These methods are time-consuming and suffer from hydrolysis inefficiency [9,10]. They also do not determine the site of conjugation [11].

Direct measurement by GC suffers from the lack of volatility and high-tem-

TABLE I

RECOVERIES OF CONJUGATED STEROIDS ADDED TO HUMAN URINE ($n = 10$)

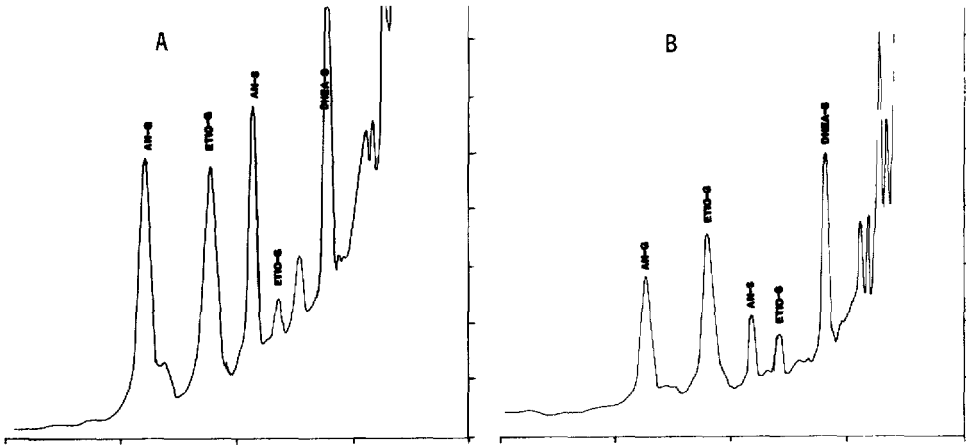| Steroid | Recovery (%) | Coefficient of variation (%) |
|---|---|---|
| Dehydroepiandrosterone sulphate | 96 | 3 |
| Etiocholanolone sulphate | 102 | 2.2 |
| Androsterone sulphate | 98 | 4 |
| Etiocholanolone glucuronide | 104 | 2 |
| Androsterone glucuronide | 101 | 2.5 |

Fig. 5. Typical chromatograms from normal human urine samples. (A) Urine from a male adult (aged 30 years); (B) urine from a female adult (aged 26 years).

perature instability of conjugates. HPLC which is highly selective and non-destructive is a logical alternative [14]. Determination of 17-oxosteroid conjugates directly by HPLC in the microgram range using refractive index was reported by Lafosse et al. [15]. Recently, a highly sensitive method using dansyl hydrazine as a prelabelling agent was used to assay the 17-oxosteroid conjugates by HPLC [12]. Prelabelling can also be done with $p$-nitrophenylhydrazine [13] which has lead to our present spectrophotometric method using 2,4-dinitrophenylhydrazine.

Chromatographic conditions for the resolution of the dinitrophenylhydrazones of the 17-oxosteroid conjugates and other absorbing substances in urine were selected from studies on mobile phase composition. Using a $C_{18}$ column, the glucuronides behaved like weak acids and were pH-dependent as in Fig. 1, whereas
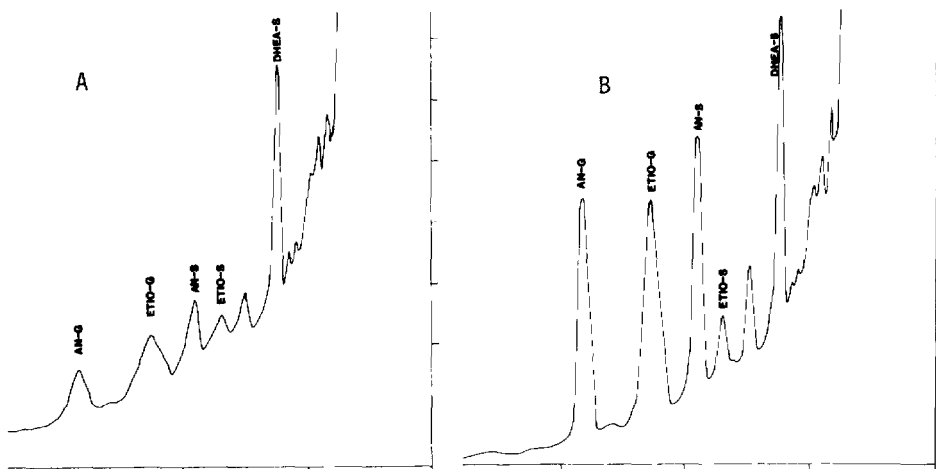


Fig. 6. Chromatograms of urine samples from a prepuberal boy (aged 9 years). (A) Pre HCG stimulation; (B) post HCG stimulation.

the sulphates behaved like strong acids and were ion-pair-dependent as in Fig. 2 [16] using $Na^+$ as the counter-ion. When the amount of sodium acetate (ion-pairing agent) was varied in the buffer there was the attendant pH change. This led to opposite retention from the glucuronides and sulphates (Fig. 2). By using less acid in the buffer there is less $H^+$ to compete with ion-pairing and so more retention of the sulphates with better separation from excess dinitrophenylhydrazine. Finally, variation in the methanol concentration in the mobile phase caused a dramatic change on retention (Fig. 3). From these studies, 70% methanol using buffer containing 2% acetic acid and 50 m$M$ sodium acetate was chosen as the optimum solvent system.

Though the fluorescence technique using dansyl hydrazine is highly sensitive, the spectrophotometric detection of the dinitrophenylhydrazones is adequately sensitive to assay urinary 17-oxosteroid conjugates. In adult urine samples all the 17-oxosteroid conjugates were easily identified. However, in urine samples from prepubertal boys, the 17-oxosteroid conjugates were more easily detected following HCG stimulation. This improves the measurement of ETIO-G/AN-G ratio when screening for $5\alpha$-reductase deficiency [2].

In conclusion, the HPLC method with spectrophotometric detection provides a direct convenient method for routine analysis of urinary 17-oxosteroid conjugates for the study of androgen metabolism.

ACKNOWLEDGEMENTS

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