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Short communication

Fluorescence derivatisation of urinary corticosteroids for high-performance liquid chromatographic analysis

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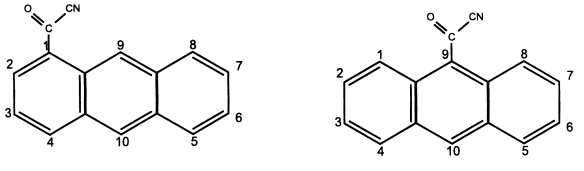
Abstract

Corticosteroids containing a C_{21} primary hydroxyl group were derivatised with 9-anthroyl cyanide. The reagent was prepared as a solution in acetonitrile, containing 0.1% triethylamine, at a concentration of 2 mg/ml. Approximately 1 μg of corticosteroid was reacted with 100 μl of this reagent, at 45°C for 2 h. The fluorescent derivatives were separated by HPLC on a silica column, 250×4.6 mm I.D., by stepwise elution, with a mobile phase of 2-propanol–hexane (2:98) for 20 min, followed by 2-propanol–hexane (7:93) from 20 to 40 min. The fluorescence detector was set to 370-nm excitation and 470-nm emission. The relatively low temperature for derivatisation avoided reaction with secondary hydroxyl groups and also prevented thermal degradation of the corticosteroids.

Keywords: Corticosteroids

1. Introduction

Although chromophoric and fluorescence-producing reagents are available for reaction with many functional groups, there are still problems with the pre-column derivatisation of aliphatic hydroxy groups for HPLC. A review on the HPLC analysis of corticosteroids, including derivatisation methods, has been published by Volin [1]. An important contribution was made by Goto et al. [2] who synthesised two isomeric anthroyl cyanides, with the functional group in the 1 or 9 position (see Fig. 1). This group reacts with aliphatic hydroxy compounds, and the anthracene nucleus provides fluorescence. Whereas the 1-isomer gave variable yields in reaction with different 3-hydroxy steroids, the 9-isomer was reported to be almost completely inert under their



Formulae of isomeric anthroyl cyanides.

Fig. 1. Formulae of isomeric 1- and 9-anthroyl cyanides.

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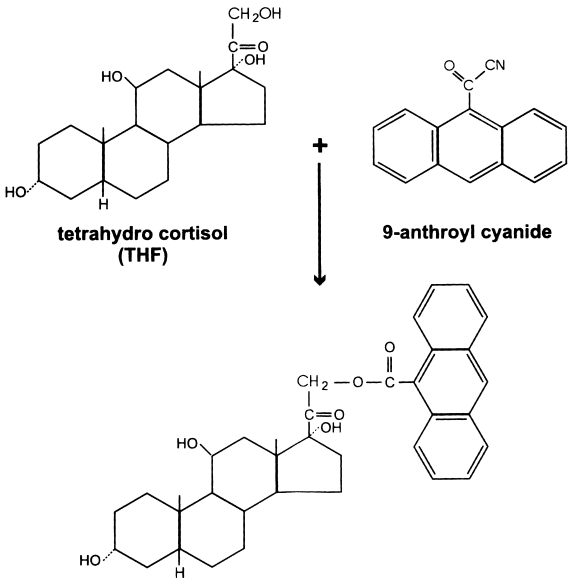

Fig. 2. Derivatisation of tetrahydrocortisol.

conditions. However, cortisol reacted readily with both isomers, presumably on the 21-hydroxy group (see Fig. 2).

We considered, therefore, that the 9-anthroyl cyanide should be a useful reagent for the analysis of urinary corticosteroids, that possess both 3- and 21-hydroxy groups. However, in our hands, a considerable degree of reaction occurred in the 3-position as well as on the 21-group. Since multiple derivatisation gives rise to a complex chromatogram, we have modified the conditions to give reaction only on the 21-hydroxy group. The use of this modification and the chromatography of the principal corticosteroids occurring in urine are the subject of this communication.

2. Experimental

2.1. Reagents and chemicals

9-Anthroyl cyanide was synthesised according to the method of Goto et al. [2] in the School of Chemistry, Tel-Aviv University (kindly provided by Professor Y. Kashman). Steroids were from Makor Chemicals (Jerusalem, Israel). Solvents used were of analytical grade, except those for HPLC which were of HPLC grade.

2.2. Derivatisation

Corticosteroid standards were prepared in ethanol solution at a concentration of 1 mg/ml and were stored at −18°C. Usually, 1–5 μg of each steroid was used for derivatisation. The ethanol was evaporated at 37°C under a stream of air. 9-Anthroyl cyanide was prepared at a concentration of 2 mg/ml in a mixture of 0.1% triethylamine in acetonitrile. A 100-μl volume of this reagent was added to the dry steroid and heated for 2 h on a heating block at 45°C. At the end of the reaction, the solvent was evaporated and the steroid derivatives were kept in the dark pending chromatography.

2.3. Thin-layer chromatography (TLC)

TLC of the steroid derivatives was performed on silica gel plates (Kieselgel 60 F_{254}, Merck, Darmstadt, Germany). Solvents employed for separation included: methanol–chloroform (4:96); dioxane–chloroform (4:96); acetonitrile–dichloromethane (25:75); ethyl acetate–dichloromethane (40:60).

2.4. High-performance liquid chromatography (HPLC)

The apparatus used was an LDC Analytical CM 4000 solvent delivery system equipped with an Econosphere silica 5 μm column, 250×4.6 mm I.D. (Alltec). The fluorescence detector was a Jasco PF-210. The excitation wavelength was 370 nm and emission 470 nm. Approximately 10 ng of each steroid derivative was injected. The compounds were eluted using a stepwise gradient of 2-propanol–hexane, 2:98 from 0 to 20 min, and 7:93 from 20 to 40 min. At the end of the run, the column was reconditioned with 2-propanol–hexane, (2:98, v/v) prior to the next run.

An even better separation was afforded by mixtures of acetonitrile–dichloromethane, with concentrations of acetonitrile around 10%, but dichloromethane cannot be used on HPLC systems that are affected by chlorinated hydrocarbons.

2.5. Analysis of urinary steroids

Urine samples (5 ml) were prepared by the method of Kraiem et al. [3], as modified by Chayen et al. [4]. Basically, this involves enzyme hydrolysis of steroid conjugates with *Helix pomatia* juice followed by extraction of the steroids with 0.5 *M* NaOH. The ether extraction and alkali wash give the parent steroids and their tetrahydro metabolites, but not cortols and cortolones. The dried extract of steroids was derivatised as described above (Section 2.2) for standards.

3. Results and discussion

3.1. Conditions for mono-derivatisation

Derivatisation of steroids by 9-anthroyl cyanide was attempted using the reaction conditions of Goto et al. [2]. The steroids tested were THDOC, THS, allo-THF, THF and THE, all of which have hydroxy groups at carbons 3 and 21, as well as at C_{11} in the case of THF and allo-THF. All of these steroids gave two derivatives as demonstrated by TLC. Reaction of 9-anthroyl cyanide with androsterone (that has one hydroxyl group at C_3) and cortisol (that has a primary hydroxyl group at C_{21} and a sterically-hindered hydroxyl at C_{11}) gave only one derivative. The reaction with androsterone indicated that our reagent reacted also with the 3α-hydroxyl group.

Inasmuch as the C_{21} hydroxy group reacts readily with the reagent, we assumed that the two derivatives of the tetrahydro steroids obtained at 60°C were the 21-mono derivative and the 3,21-diderivative.

When derivatisation was performed at lower temperatures, only one spot was obtained on TLC. This was the lower spot, and should be the C_{21} derivative. The final procedure chosen for derivatisation was 2 h at 45°C. Under these conditions, cortisol reacted but androsterone did not.

3.2. Separation of the derivatives by HPLC

Separation of the steroid derivatives under normal-phase conditions using a silica column (as described in Section 2.4) gave well-defined peaks, and the steroids were well separated from one another. Each reaction mixture, as well as the reagent blank, also gave a fairly wide peak with low retention time, but this peak eluted well before the appearance of the first steroid peak, the THDOC derivative.

Various synthetic steroids were tried as internal standard, and the most suitable was flurandrenolide, both from the point of view of retention time and recovery. In the case of urine samples, in which the steroid conjugates have to be subjected to enzyme hydrolysis, the internal standard was added prior to hydrolysis.

Fig. 3 shows the separation of derivatives of standard corticosteroids. Fig. 4 is a chromatogram of a derivatised urine extract (following enzyme hydrolysis in the presence of the internal standard). Fig. 5 is a similar chromatogram from a case that we had

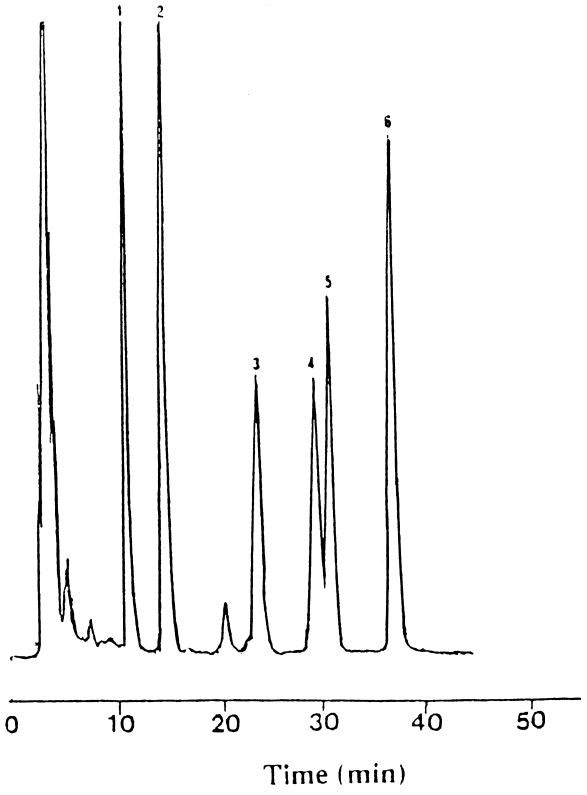

Fig. 3. Chromatogram of standard steroids: THDOC (1), flurandrenolide as internal standard (2), THS (3), allo-THF (4), THF (5), THE (6). The derivatised steroids were dissolved in 200 μl of hexane and 10 μl was injected. The eluting solvent was 2-propanol–hexane, 2:98 (v/v), changing to 7:93 (v/v) at 20 min.

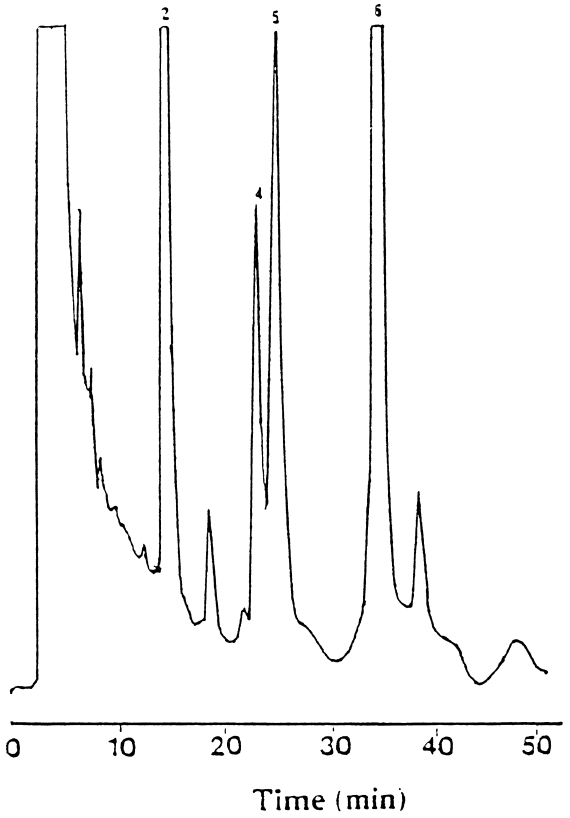

Fig. 4. Chromatogram of steroids from a normal urine. Conditions for HPLC were as in Fig. 3.

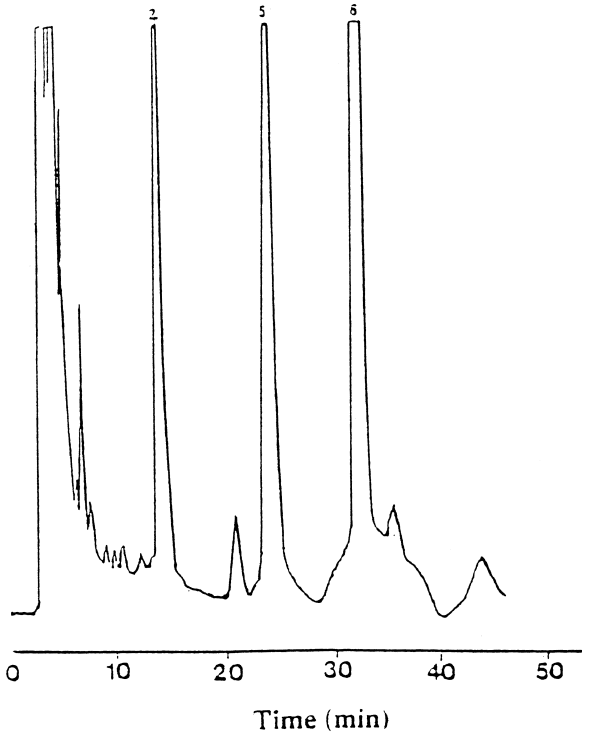

Fig. 5. Chromatogram of steroids from urine of a patient suffering from 5α-reductase deficiency. Note the absence of allo-THF. Conditions for HPLC were as in Fig. 3.

demonstrated to be one of 5α-reductase deficiency [5]. In the latter, allo-THF is absent.

The main difference in the present use of anthroyl cyanide from that developed by Goto et al. [2] is the reduction of temperature to 45°C for derivatisation. This ensures that only the C_{21} hydroxyl group will react. As stated by Goto et al. [2], the reagent will not react with the tertiary hydroxyl at C_{17} nor with the sterically-hindered hydroxyl at C_{11}. The selection of the 9-isomer of anthroyl cyanide further promotes steric hindrance, and the use of the lower reaction temperature finally makes the derivatisation selective for the primary C_{21} hydroxy group. The C_3 hydroxy does not react at the lower temperature, so that the urinary corticosteroids each give only a single peak in HPLC.

The present method measures free steroids and their tetrahydro metabolites. The latter are important in the diagnosis of metabolic disorders such as the 11β-hydroxylase block (congenital adrenal hyperplasia) and enzyme deficiencies of 5α-reductase and 11β-hydroxysteroid dehydrogenase, as well as Cushing's syndrome.

9-Anthroyl cyanide is a stable reagent when kept desiccated in the dark, and the sensitivity provided by the strong fluorescence is such as to permit analysis of corticosteroids in 1 ml of urine.

4. Abbreviations

TH.DOC	(Tetrahydrodeoxycorticosterone) 3α,21-dihydroxy-5β-pregnan-20-one
THS	(Tetrahydro-11-deoxycortisol) 3α,17α,21-trihydroxy-5β-pregnan-20-one
THF	(Tetrahydrocortisol) 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one

allo-THF	(allo-Tetrahydrocortisol) 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one
THE	(Tetrahydrocortisone) 3α,17α,21-trihydroxy-5β-pregnan-11, 20-dione
Flurandrenolide	6α-Fluoro-11β, 16α, 17, 21-tetrahydroxy-pregn-4-ene-3, 20-dione 16, 17-acetonide

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