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Note

Selectivity of a diol phase high-performance liquid chromatographic system in trace analysis of anabolic compounds

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In the Netherlands the control for the illegal use of hormonal anabolics in cattle has been focused for the past few years on the presence of the "stilbenes" diethylstilbestrol (DES), dienestrol (DE) and hexestrol (HEX) in urine. Detection methods have been developed, including radioimmunoassay (RIA)[1], thin-layer chromatography (TLC)[2] and gas chromatography–mass spectrometry (GC–MS)[3]. To increase both the specificity and the sensitivity by a substantial purification of the urine extract, a selective reversed-phase high-performance liquid chromatographic (HPLC) step was introduced[4] prior to all detection techniques.

The same isocratic HPLC system was also used in an investigation of illegal preparations isolated from application sites of slaughtered cattle[5]. In this multi-residue monitoring study, ten different anabolic compounds were found more or less frequently. Because of the high concentrations and relatively clean matrix, on-line detection with a diode array was possible and turned out to be very specific for identification purposes also. For monitoring and screening purposes in urine, however, there is still an urgent need for fast multi-residue detection methods using HPLC–UV, TLC or GC–MS. The HPLC clean-up with reversed-phase chromatography was not satisfactory. On testing other HPLC columns, which were described in the literature for separation of androgenic[7,8] and/or estrogenic[8–12] steroids, it was found that normal-phase HPLC columns substantially improved the purification of steroids from urine samples[6].

A practical and simple prepurification step that uses a diol phase HPLC column, in combination with various detection methods, is presented in this report.

MATERIALS AND METHODS*

The HPLC equipment consisted of an automatic injector (WISP, Waters Assoc.), a solvent delivery system (Model 2150, LKB), a variable-wavelength detector

* Reference to a company and/or product is for purposes of information and identification only and does not imply approval or recommendation of the company and/or the product by the National Institute of Public Health and Environmental Hygiene, to exclusion of others which may also be suitable.

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operated at 210 nm (Model 773, Kratos) and a printer–plotter–integrator (Model 3390A, Hewlett-Packard). The chromatographic column (150 mm × 4.6 mm I.D.), obtained from Chrompack, was packed with LiChrosorb Diol, 5 μm (Merck) using a Column Packing Instrument (Shandon). Elution conditions were isooctane–ethanol (95:5, v/v or 93:7, v/v) at a flow-rate of 2.0 ml/min.

All solvents were of analytical grade (Merck). Anabolic standards were checked for purity by melting point, HPLC and infrared spectroscopy.

RESULTS AND DISCUSSION

Characteristic retention times of some endogeneous steroids and some frequently found anabolics on a LiChrosorb Diol (5 μm) column are listed in Table I, from which it can be seen that the anabolic compounds and some of their metabolites can be divided into two groups. From 2 to 7 min the androgens methyltestosterone (MT), testosterone (T), 19-nortestosterone (NT) and Trenbolone (TB), and the gestagens progesterone (P) and medroxyprogesterone (MP), are eluted and from 10 to 15 min the phenolic estrogens estradiol (E), *meso*-HEX, Zeranol (Z), ethynylestradiol (EE_2), *trans*-DES and alpha-DE.

TABLE I

RETENTION TIMES OF VARIOUS ANABOLIC COMPOUNDS AND SOME OF THEIR METABOLITES ON A COLUMN OF LICHROSORB DIOL

Compound	t_R *(min)*
Progesterone (P)	2.1
17α-Methyltestosterone (MT)	3.7
17α-Testosterone (α-T)	4.4
Medroxyprogesterone (MP)	4.5
17β-Testosterone (T)	4.6
19-Nortestosterone (NT)	5.1
17β-Trenbolone (TB)	6.5
17α-Trenbolone(α-TB)	6.7
17α-Estradiol (α-E_2)	10.0
17β.Estradiol (E_2)	10.4
meso-Hexestrol (HEX)	12.0
Zeranol (Z)	12.4
17α-Ethinylestradiol (EE_2)	12.5
trans-Diethylstilbestrol (DES)	13.3
α-Dienestrol (DE)	14.4

For practical purposes the composition of the mobile phase can be changed in order to collect the androgen–gestagen and estrogen fractions in a smaller volume (Fig. 1). In addition, this HPLC step causes a substantial purification of the urine extract, since the majority of the matrix components are retarded on normal-phase columns[6].

The separation in an androgen–gestagen fraction and an estrogen fraction is of great importance for the final detection methods. For two-dimensional TLC analy-

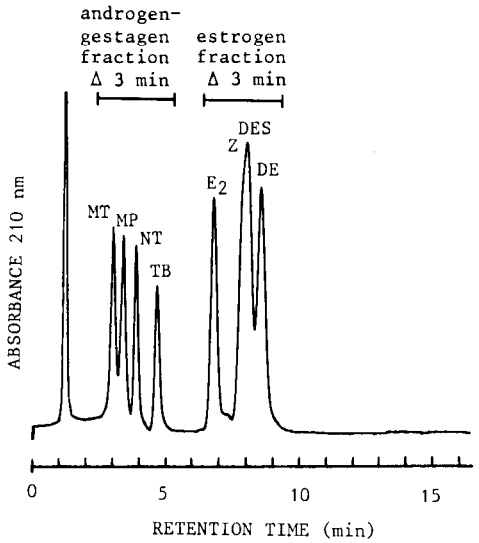

Fig. 1. HPLC group separation of a standard mixture on a LiChrosorb diol column after elution with isooctane–ethanol (93:7, v/v) at a flow-rate of 2.0 ml/min. The total volume of the combined androgen–gestagen and estrogen fractions is 6.0 ml each under these conditions.

sis both the elution system and the detection system methods are different for both groups of anabolics. Androgens are generally detected on the TLC plate by *in situ* fluorescence after acid treatment[13], whereas the estrogens E_2, HEX, Z and EE_2 are also convertable into their dansyl derivatives prior to TLC analysis[14].

For on-line UV detection the separation between androgens and estrogens has a very practical application. In subsequent HPLC analysis, the androgens and gestagens can be detected in general most sensitively at their maximum absorbances (λ_{max} 242–244 nm), whereas the estrogens have their maximum absorbances at substantially lower wavelengths (195–215 nm). 3-Ketotrienic steroids such as trenbolone are best detected at 350 nm.

Another application in which the separation between the two groups is very important for further analysis is GC–MS. The derivatization to trimethylsilyl derivatives can be performed on estrogens without further precautions. For androgens, however, the 3-keto steroids have to be converted into their 3-carboxymethoxime derivatives prior to derivatization of the 17-hydroxyl groups.

In this report a fast and simple separation method is presented for the fractionation of androgen and gestagen anabolics and estrogen anabolics, which has implications for the use of final detection methods, Such as TLC, UV and GC–MS analysis. Further work is in progress to integrate this purification method into the routine detection procedures of hormonal anabolics in urine.

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