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ANALYSIS OF STEROID MIXTURES BY COLUMN CHROMATOGRAPHY WITH CONTINUOUS MONITORING OF THE ELUATE BY A FLAME IONISATION DETECTOR

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SUMMARY

A method is described for the separation of steroids in mixtures of pharmaceutical interest by means of silicic acid column chromatography. Associations of estrogenic with progestational steroids, in some cases extended to androgens, are considered.

Elution is performed with a gradient of ethyl ether in petroleum ether, obtained by means of two metering pumps. The effluent is monitored continuously by means of a hydrogen flame ionisation detector, a part of the effluent being continuously drawn off to the detector.

The quantitative analysis of the steroids, separated as described in the method, is performed by a UV spectrophotometric or a colorimetric procedure.

INTRODUCTION

Interest in separation methods for steroids in pharmaceutical preparations is still increasing, parallel with the increase in the number of new compounds and complex formulations used in therapy.

Most papers deal with thin-layer chromatography (TLC) techniques, owing to their simplicity and variety, but in many cases column chromatography techniques are still useful, especially for isolation and determination purposes.

In a previous paper[1], we described a method for the fractionation of lipids into classes by means of silicic acid column chromatography; this method was characterised by a good degree of automation, reproducibility and simplicity.

During the first experiments concerning wider applications of our method, we observed promising possibilities for the separation of steroids from the components of an oil base and in many cases also of different steroids from each other. Our interest in the general problem of the separation and determination of steroids in oil solutions for pharmaceutical use has been summarised in a previous paper of this series[2].

The analysis of steroids in mixtures of two or three components was a logical extension of the application of our procedure. The present study on the separation of steroid mixtures concerns principally estrogens and progestins, in some cases associated with androgens. Many papers have been published on this subject, especially

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using TLC techniques[3–7]. A recent paper by ALVAREZ FERNANDEZ AND TORRE NOCEDA[8] describes the separation of progestins and estrogens by means of column chromatography on Sephadex LH-20 with methanol–water (85:15, v/v) as eluant; our results compare favourably with those reported by these authors, especially regarding better separations between different progestins.

EXPERIMENTAL

The major details of the experimental methods have been published earlier[1], and only a few modifications are described in detail in this paper.

Solvents and materials for column chromatography

Petroleum ether for chromatography, boiling range 65–75°, and ethyl ether, peroxide-free, were both processed as described earlier[1]. Silicic acid, Biorad 325 mesh for lipid chromatography, according to Hirsch and Ahrens, was used, conditioned for water content as described earlier[1].

Chromatographic columns

LKB glass columns, type 4200, 6 mm in diameter and 60 cm in length, were used. Connections between the columns and other parts of the apparatus were made with Teflon tubes, O.D. approx. 1/16 in. (1.58 mm). The load of silicic acid was 10 g to obtain a filling height of 56 cm.

Sample preparation and introduction into the column

The compounds used are listed in Table I. They were pure for pharmaceutical use and analysed by UV spectrophotometry when used for quantitative determinations. The absorptivity values, *a*, used in the determinations were as follows: testo-

TABLE I

ELUTION VOLUMES FOR THE ANALYSED STEROIDS

Steroids	*Elution volume (ml)*
Mestranol	255
Estradiol 3-benzoate	365
Ethynyl estradiol	385
Allylestrenol	145
Lynestrenol	165
Ethylestrenol	165
Ethinodiole diacetate	260
Vinylestrenolone	365
Norethynodrel	380
Chlormadinone acetate	>440[a]
Norethindrone	>440[a]
Progesterone	>440[a]
Testosterone propionate	355
Methyltestosterone	>440[a]
Tocopherol acetate	110
Tocopherol	125

[a] Elution with 100% ethyl ether.

sterone propionate, 49.2 at the maximum of 240 nm; estradiol benzoate, 51.8 at the maximum of 230 nm; mestranol, 6.6 at 278 and 6.2 at 287 nm maxima, respectively, in accordance with the values reported in Ref. 9.

Heptane or benzene–heptane solutions of the analysed steroids were prepared at a concentration of 1 or 2 mg/ml.

Feeding system for the column

This system is described in detail in Ref. 1; the operating conditions for the gradient are reported in the legends to Figs. 1–4.

Analysis of eluates

The operating conditions for the Liquid Chromatography Detector (LCD), Mod. 5400 of Barber Colman, were as reported in Ref. 1. The splitting ratio was adjusted to send 6–7 % of the total effluent volume to the detector when the flow rate, R_2, was 1 ml/min. The fraction collector was regulated to fractions of 10 min.

The spectrophotometric determination of the UV-absorbing steroids was performed by collecting the total volume, or its measured aliquot, of all the fractions corresponding to the peak, in a tared vessel of 25 or 50 ml and adjusting to the mark with petroleum ether. A convenient aliquot (5 ml or less if the analysed quantity is 1 mg or more; 10 ml if this quantity is 200 μg; the total volume if the steroid has a poor absorptivity value, as in the case of mestranol) was twice taken from this solution and evaporated to dryness, under nitrogen flow in tared vessels of 10 ml, heated in an aluminium block thermoregulated to 40°. The evaporation was performed under controlled conditions, maintaining the nitrogen flow through the vessel for 15 min after the end of the evaporation. Each residue was removed with ethanol, adjusting the volume to the mark. The UV spectrum was plotted in the range corresponding to the maximum for the analysed steroid. A Beckman DU-2 spectrophotometer was used with 1-cm cuvettes. The quantity of steroid recovered was calculated using the following equation:

$$\frac{\bar{A}_M}{a} \cdot 1000 \cdot D = Q_R$$

where Q_R = quantity of steroid recovered in the peak; $\bar{A}_M$ = mean value of the measured absorbance, a = absorptivity (absorbance index for a solution at a concentration of 1 g in 1000 ml), D = dilution.

Referring Q_R to the sample quantity, Q_C, corrected for Q_S, the quantity split to the LCD, or more generally corrected for the percentage aliquot, A, of the eluate volume taken for the analysis, the percent recovery $R = (Q_R/Q_C') \cdot 100$ is obtained, where $Q_S = Q_C \cdot S$ and S is the percent splitting value; $Q_C' = Q_C - (Q_C \cdot S)$ or $Q'_C = Q_C \cdot A$.

The colorimetric determination of mestranol was performed as described by TISILIFONIS AND CHAFETZ[10].

RESULTS AND DISCUSSION

As regards the separation of complex formulations of steroids with estrogenic and

progestational activity, in some cases extended to androgens, we report, as examples, the analysis of the following mixtures of pure steroids:

(1) Mestranol (0.5 mg), norethynodrel (0.5 mg) and norethindrone (20 mg). Fig. 1 shows the clear separation of the three peaks, also in the presence of an excess of norethindrone.

(2) Norethynodrel (1 mg), ethynodiol diacetate (1 mg) and methyltestosterone (1 mg). Fig. 2 shows the clear separation of the three peaks.

(3) Allylestrenol (1 mg), lynestrenol (1 mg), ethylestrenol (1 mg), estradiol benzoate (2 mg), ethynylestradiol (1.5 mg) and norethindrone (1 mg). Fig. 3 shows the

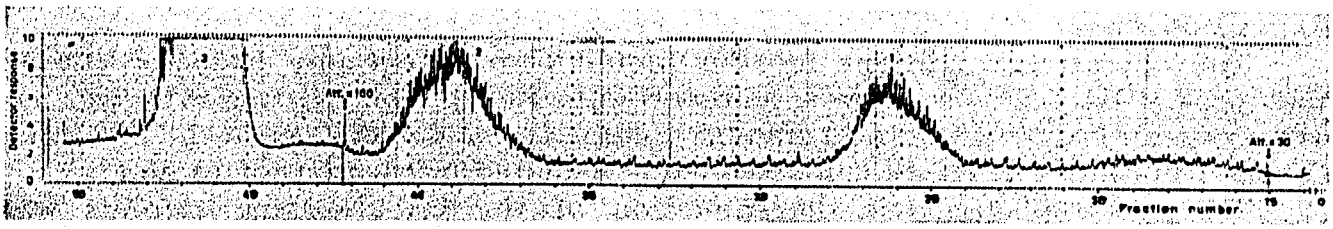

Fig. 1. Chromatogram of a mixture of mestranol, norethynodrel and norethindrone. Sample: 1 ml of a chloroform solution containing 0.5, 0.5 and 20.0 mg, respectively, of the above steroids. Operating conditions: column, 6 mm diameter × 560 mm high, charged with 10 g of silicic acid. Detector sensitivity at 9×10^{-11} (att. × 30) from fraction 16–42 and at 3×10^{-10} (att. × 100) from fraction 43 to the end of the run. Concave gradient $P = 1/4$; C_R = 100% ethyl ether, 86 ml; C_M = 100% petroleum ether, 344 ml (plus 30 ml before the gradient). Column flow rate, 1 ml/min. Splitter ratio, 6.5% of the column flow to the detector. Chart speed, 30 cm/h (≃ 12 in./h). Peaks: 1 = mestranol; 2 = norethynodrel; 3 = norethindrone.

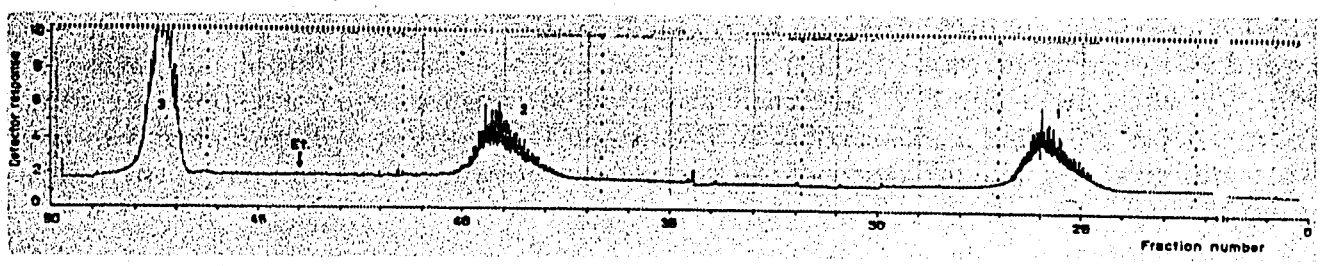

Fig. 2. Chromatogram of a mixture of methyltestosterone, norethynodrel and ethynodiol diacetate. Sample: 1 ml of a benzene–heptane solution (10:90, v/v), containing 1 mg of each steroid. Operating conditions as in Fig. 1 except detector sensitivity at 3×10^{-10} (att. × 100). Peaks: 1 = ethynodiol diacetate; 2 = norethynodrel; 3 = methyltestosterone.

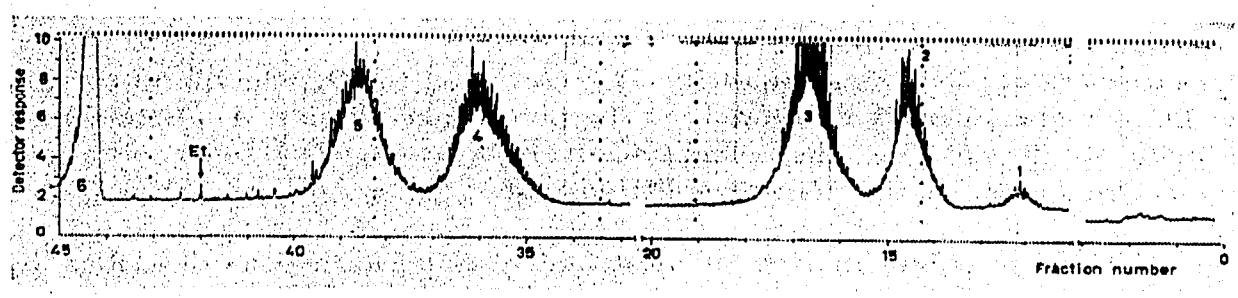

Fig. 3. Chromatogram of a mixture of allylestrenol, lynestrenol, ethylestrenol, estradiol benzoate, ethynylestradiol and norethindrone. Sample: 1 ml of a benzene–heptane (35:65, v/v) solution, containing 1, 1, 1, 2, 1.5, and 1 mg, respectively, of the above steroids. Operating conditions as in Fig. 1, except detector sensitivity at 3×10^{-10} (att. × 100). Peaks: 1 = triglycerides (in small amount as impurities); 2 = allylestrenol; 3 = lynestrenol + ethylestrenol; 4 = estradiol benzoate; 5 = ethynylestradiol; 6 = norethindrone.

good separation of five of the six components, peak no. 3 being due to the non-resolved lynestrenol and ethylestrenol.

These mixtures do not correspond to real pharmaceutical associations, but were formulated for investigation and demonstration purposes: the chromatographic behaviour and the separation possibilities of the steroids studied are summarised in Table I, in which are reported the elution volumes of the listed compounds under the operating conditions described in the text. As is shown in Table I and by the reported examples, the separation possibilities are generally good. For other hormonal steroids, additional values of elution volumes obtained under similar conditions are reported in Ref. 2. This demonstrates satisfactory efficiency of the columns and correct calculation of the operating conditions. It should also be considered that in this investigation only the gradient of ethyl ether in petroleum ether was employed; different gradients may be investigated and other steroid mixtures can probably be resolved.

An example of the quantitative analysis of some steroids, separated by the present method, is given in Table II. The results demonstrate satisfactory recovery and precision (the latter being expressed as standard deviation), considering the reduced quantities used for the analysis; better recovery is obtained on 1 mg samples[2]. On the other hand, in the analysis of the estrogenic component of the estro-progestinic

TABLE II

DETERMINATION OF PERCENTAGE RECOVERY

Sample	*Quantity of chromatographed steroid (mg)*	*Recovery (% ± S.D.)*	
		Spectrophotometric	*Colorimetric*
Testosterone propionate	0.200	94.0 ± 3.4 (4)[a]	—
Mestranol	0.200–0.400	92.3 ± 2.2 (6)[a]	94.5 ± 3.2
Estradiol benzoate	0.200	93.7 (2)[a]	—

[a] Number of determinations.

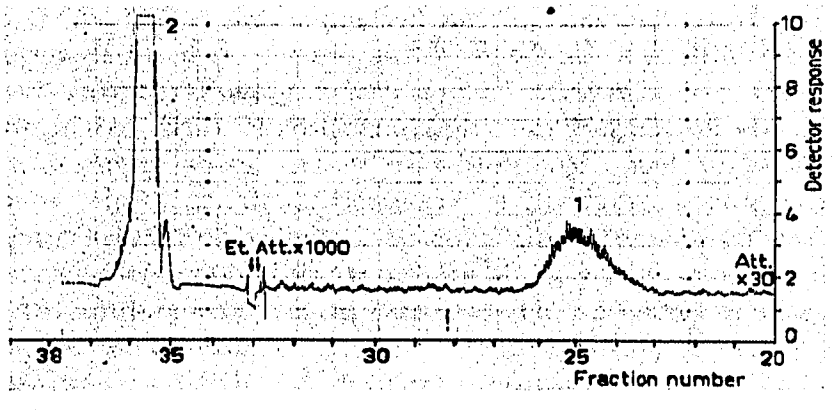

Fig. 4. Chromatogram of a sample of norethindrone, 20 mg, with mestranol impurity. Operating conditions as in Fig. 1, except (a) detector sensitivity at 9×10^{-11} (att. × 30) from fraction 1–33 and at 3×10^{-9} (att. × 1000) from fraction 33 to the end of the run, and (b) chart speed 6 in./h. Peaks: 1 = mestranol, estimated as 1.2% of the chromatographed amount; 2 = norethindrone

complex formulations, the quantities of the estrogen that have to be dealt with are often necessarily small, owing to its low percentage content in the mixture.

A final feature to be pointed out is the possibility of overloading a column with a substance or mixture sample, in order to isolate a component present at low concentration for its analysis or, if this component is an impurity, for its characterization. As an example, Fig. 4 shows a chromatogram of norethindrone, 20 mg, containing mestranol as an impurity. The estrogen was analysed in the peak eluate by spectrophotometric and colorimetric methods and found to be as high as 243 μg, corresponding to 1.2 % of the analysed norethindrone. The identity of mestranol was ascertained by means of TLC, by UV and sulphuric acid–methanol chromogen spectra and by means of a second column chromatography, adding to the sample an authentic specimen of mestranol; the peak was reinforced and recovery confirmed the previous value.

In conclusion, we can say that the chromatographic method described is useful both for analytical and for isolation purposes. Compared with TLC methods it is obviously less simple but is more amenable to automation. In addition, it permits one to analyse more consistent samples and to use more than one technique for determinations or characterisations.

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