QUANTITATIVE SEPARATION OF STEROIDS IN OILY SOLUTIONS BY MEANS OF THIN-LAYER CHROMATOGRAPHY WITH CONTINUOUS ELUTION

G. CAVINA AND G. MORETTI
Istituto Superiore di Sanità, Rome (Italy)
(Received September 10th, 1965)

In previous work conducted in this laboratory on microtechniques applicable to the isolation and determination of steroids possessing hormonal activity in the presence of lipid organ extracts, a description of a method of separation and determination of corticosteroid and lipid constituents present in the total lipid extract of rat adrenal glands using thin-layer chromatography was given[1].

Further studies dealing with the extension of this technique to the general problem regarding the separation of steroids less polar than corticosteroids, such as progesterone, testosterone and 19-nortestosterone esters and estrogens, from mixtures of lipids such as incubates, organ extracts and oily solutions, resulted in a method for the isolation and determination of some steroid hormones in oily solutions used for pharmaceutical purposes.

The methods described in the literature for the separation of steroids in such oily pharmaceutical preparations are based on physico-chemical separations, such as countercurrent extraction with ethanol and hydrocarbons[2,3], adsorption chromatography on Florisil as described by UMBERGER[4] and TAPPI *et al.*[5], or partition chromatography using either ethanol on ground silica and iso-octane[2], or polyethylene glycol 600 on ground silica and hexane[6] or nitromethane on ground silica and heptane[7]. These methods have generally been described for individual problems.

Some applications of paper chromatography to the separation of steroids in oily solutions have also been reported, although they are less numerous than those described for other pharmaceutical forms or for purity controls[8-11]. ROBERTS AND FLOREY[12] have described the determination of long chain esters of testosterone and 17α-hydroxyprogesterone in concentrated oily solutions (125–250 mg/ml) using paper chromatography followed by quantitative determination with isonicotinic hydrazide reagent in acid methanol as described by UMBERGER[4] using the same reagent as the means of elution.

A similar technique[13] to that for the determination of the 3-cyclopentyl enol ether of progesterone (Quingesterone) in oily solutions in the presence of its transformation products[14], has recently been described for the separation and determination of 19-nortestosterone decanoate (nandrolone decanoate) from oily solutions (concentration 50 mg/ml).

Thin-layer chromatography has been applied to the quantitative analysis of steroids of various degrees of purity[15], to corticosteroids and natural cortical extracts[16],

to the determination of 17α-ethynyl-estradiol 3-methyl ether in the presence of synthetic progesterones and compared with gas chromatographic analysis[17], to the determination of 17β-hydroxy-Δ^{4}-estrene-17α derivatives in tablets[18] and to the analysis of 6-chloro-17α-hydroxypregnane-4,6-diene-3,20-dione acetate, also in mixtures with 17α-ethynylestradiol 3-methyl ether[19].

Applications of this technique to the analysis of steroids in oily solutions are only qualitative. KORZUN AND BRODY[20] have described the identification of some steroids of pharmaceutical interest using various colorimetric reactions after silica gel thin-layer chromatography using various solvents which permitted separation from a greater part of the oily solvent.

Our studies have been directed towards the development of a continuous elution technique for ascending thin-layer chromatography that is at once simple and effective, and permits the complete separation of the steroids not only from triglycerides, but also from the minor components of the oils such as diglycerides and free fatty acids that cannot be separated from the steroids under the normal conditions of chromatography, and also allows the spectrophotometric determination in the U.V. of the steroids with a satisfactory recovery.

The eluates were obtained in high purity conditions and identification was possible throughout the entire U.V. spectrum. It is also possible to apply other reactions for the determination such as that with isonicotinic hydrazide.

EXPERIMENTAL*

Preparation of the standard solution and samples

Steroids purified for pharmaceutical use were employed in the preparation of solutions in spectrograde ethanol at a concentration of 5 mg/ml (250 mg/50 ml).

The compounds used for quantitative analysis included testosterone propionate, progesterone, 19-nortestosterone propionate, and estradiol cyclopentylpropionate. Solutions of testosterone cyclopentylpropionate and estradiol benzoate at the same concentration were also used for the qualitative analysis.

The absorbance index in ethanol (absorptivity as described by *Chemical Abstracts*, $E_{1\,cm}^{1\%}$ according to previous denomination) was determined for each steroid by diluting the 5 mg/ml ethanol solution. Samples were withdrawn with a Hamilton microsyringe, tared to 50 μl and diluted to 25 ml with ethanol. The values obtained were in good agreement with the values reported in the literature[21,22]. To obtain greater exactness of the chromatography recovery values, the values of the absorbance index as determined above were used. They were 491 for testosterone propionate and 535 for progesterone.

The steroids so analysed were used for the preparation of solutions in pure olive oil for pharmaceutical use with the addition of 1 % benzyl alcohol. The solutions prepared for the quantitative tests were the following:

(1) testosterone propionate (25 mg/ml);
(2) progesterone (10 mg/ml);
(3) 19-nortestosterone propionate (10 mg/ml);
(4) estradiol cyclopentylpropionate (5 mg/ml).

---

* The experimental work was carried out in collaboration with A. MOLLICA.

Thin-layer chromatography

20 × 20 cm plates coated with Merck Silica Gel G washed in a Soxhlet for 24 h with chloroform and mixed with 0.3 % Dupont Luminescent Chemical 609 were used. The thickness was 0.5 mm; activation was carried out for 30 min at 105°.

The following mixtures were used as solvents:

(1) petroleum ether b.p. 65°–peroxide-free ethyl ether–acetic acid (70:30:1);

(2) petroleum ether b.p. 65°–peroxide-free ethyl ether–acetic acid (50:50:1).

Glass tanks 18 × 25 × 22 cm fitted with smaller internal glass vessels having capacities of 100 ml for the solvents were used for the continuous chromatography. The inner vessels were placed on glass supports 5 cm from the bottom in such a manner that the upper edge of the plate, tilted at about 50° with respect to the base, was a few mm under the cover of the tank. The samples were applied as single spots or as a wide band on a line 3 cm from the lower edge of the plate. Continuous elution was carried out by placing 4 strips of Munktell 20 paper 3.5 × 20 cm* and another one of 15 × 20 cm on the upper edge of the plate. The strips were held firmly in place by means of a 20 × 5 cm glass slide and two clamps. The strips were so pressed on to the layer over an area of 3.5 × 20 cm at the upper edge of the plate. In this way, absorption of most of the triglycerides into the four 3.5 × 20 cm sheets was possible. The 15 × 20 cm sheet acted as an extension of the plate with the purpose of syphoning the solvent out of the container so as to allow continuous elution.

The prepared plate, described above, was so arranged in the tank as to allow most of the filter paper (20 × 15 cm) to pass through a narrow slit left between the cover and edge of the tank.

Solvent was put in the inner vessel which contained the lower part of the plate and the chromatogram was allowed to develop for the necessary time. In these experiments solvent 1 was run for 8 h and solvent 2 for 3 h. Under these conditions the bands of the various steroids ran mean distances of about 3.5–7.5 cm. They were clearly separated from the bands of the two diglyceride isomers (1,2 and 1,3), which ran for 10.2 and 11.3 cm (the distance is calculated from the starting line to the central points of the bands).

Quantitative separation of the steroids from oily solutions

The steroids were prepared in oily solutions as described above. They were diluted with heptane for the purpose of making them more fluid and to reduce the concentration of the steroids to 5 or 2.5 mg/ml. A Hamilton micro-syringe set at 50 µl or 100 µl was used for the deposition. The sample was distributed in small drops of equal size along a continuous line of 7 cm.

250 µg of steroids were placed on each line. On every 20 × 20 cm plate it was possible to chromatograph in parallel either two samples or one sample and a control sample of oil or the adsorbent. The heptane dilutions and the amount of oil to be separated by chromatography are shown in Table I.

Plates were developed with solvent 1 for 8 h (1st system) or with solvent 2 for 3 h (2nd system). They were then examined under U.V. light at a wavelength of 254 mµ and the zones corresponding to the steroids were marked together with one at the same height for the control.

---

* Other types of chromatography paper that are thick and porous can also be used.

TABLE I

HEPTANE DILUTIONS OF OIL SOLUTIONS OF STEROIDS

Sample	*Concentration (mg/ml)*	*Dilution with heptane, v/v*	*Quantity of steroids in 50 µl (µg)*	*Quantity of oil in 50 µl (µl)*
Testosterone propionate	25	1:5	250	10
Progesterone	10	1:2	250	25
19-Nortestosterone propionate	10	1:2	250	25
Estradiol cyclopentylpropionate	5	1:2	125	25

Quantitative separation of the steroids from alcoholic solutions

Solutions of pure steroids, concentration 5 mg/ml, were used for the purpose of determining the absorbance values of the solutions obtained by elution of the steroid band. This was done so as to serve as a reference for the determination of the recovery from the oily solutions.

As described above, 50 µl of steroid solution, concentration 5 mg/ml, were placed on each plate using a Hamilton microsyringe. An empty zone was left at the side for the control test of the adsorbent.

The plates were developed and examined with U.V. light as described previously. Five or six tests were done for each steroid and each variation in the chromatographic conditions so that values for the mean absorbance, standard deviation and average error could be obtained.

Elution and spectrophotometric determination in the U.V.

The marked areas which corresponded to the zones of migration of the steroids and the controls were carefully scraped off and the silica gel was quantitatively transferred to 50 ml separating funnels equipped with teflon stopcocks. 10 ml of water were added and the suspension was extracted four times using 5 ml of chloroform each time. These extracts were then filtered through anhydrous sodium sulfate and brought to a volume of 25 ml. 10 ml were removed and dried with nitrogen in graduated test tubes and then brought again to a volume of 10 ml with spectrograde ethanol. The spectrophotometric measurements were carried out over the range 225–250 mµ, particular attention being given to the maximum at 240–242 mµ. A Beckman DU spectrophotometer was used with 1 cm thick cuvettes. The quantity of steroid recovered was calculated using the following formula:

$$\frac{A_m - A_b}{a_{\text{det.}}} \times 10{,}000 \times 25 = \mu\text{g of steroid eluted}$$

where A_m was the steroid absorbance, A_b the blank control absorbance and $a_{\text{det.}}$ ($E^{1\%}_{1\,\text{cm}}$) was the value for the absorptivity obtained in the recovery tests using the steroid in alcoholic solution.

Determination by means of the reaction with isonicotinic hydrazide

The reagent consisted of 0.1 g of isonicotinic hydrazide and 0.125 ml of concentrated HCl in 100 ml of methanol as described by UMBERGER and has been widely used in this laboratory[23,24].

Five ml aliquots of the chloroform solution obtained in the extraction of the silica gel as described above were brought to dryness in glass stoppered test tubes; then 2 ml of methanol were added and, after shaking, 2 ml of the reagent. The solutions were protected from light and left for 30 min at room temperature. Absorbance at 380 mμ was determined against the reagent controls.

The formula used for the calculations is analogous to that used for the spectrophotometric determinations:

$$\frac{A_m - A_b}{a_{\text{det.}}} \times 10{,}000 \times 4 \times \frac{25}{5} = \mu\text{g of steroids eluted}$$

where A_m is the steroid absorbance, A_b is the blank control absorbance, $a_{\text{det.}}$ ($E^{1\%}_{1\,\text{cm}}$) is the value for the absorptivity obtained in the recovery test using the steroids in alcoholic solution and referred to the reaction mixture volume (4 ml) while 5 ml is the aliquot taken and 25 ml the total volume of the eluate.

RESULTS AND DISCUSSION

The continuous elution technique

A solution of testosterone propionate in olive oil at a concentration level of 25 mg/ml, diluted 1:5 with heptane, can be submitted to thin-layer chromatography with a petroleum ether (b.p. 65°)–ethyl ether–acetic acid (70:30:1) mixture; the steroid ranging in quantity from 100–250 μg and the oil varying between 2.5–10 mg.

If normal operating conditions as described by STAHL are followed, *i.e.* the solvent is allowed to rise to about 15 cm from the starting line, and the development time being about 45 min, it is possible to separate most of the triglycerides from the steroids, which, however, move together with the less mobile band of the diglycerides (Fig. 1a). This type of separation may be useful for the identification, but quantitative determination will not be precise because of the high and variable values of

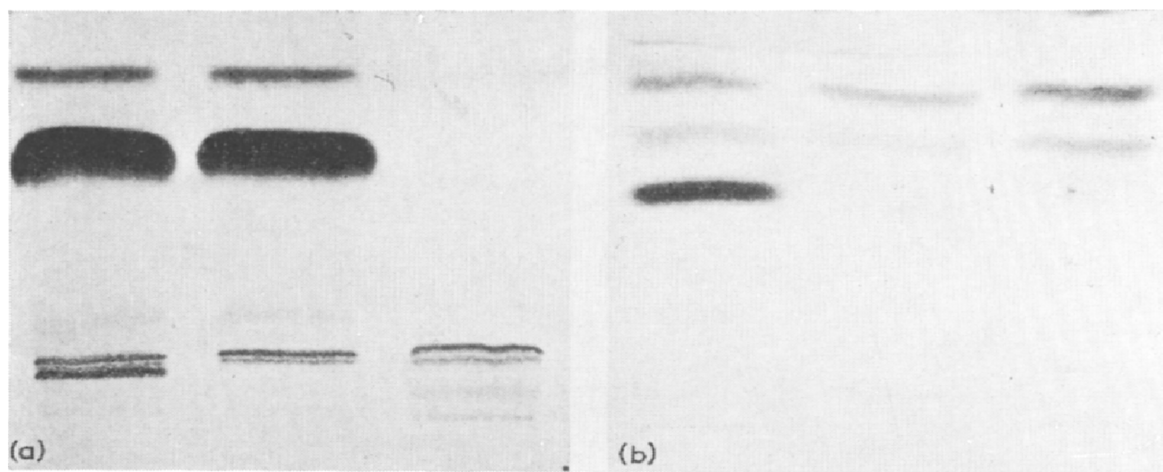

Fig. 1. (a) Chromatogram with solvent 1, development for 45 min. From left to right: testosterone propionate, oily solution; reference oil; diglyceride mixture. From bottom to top: monoglycerides (traces), two bands of diglycerides, free fatty acids, triglycerides, sterol esters. Detection: sulfuric acid. (b) Chromatogram with solvent 1, continuous development for 8 h. From left to right: same sample disposition as in (a). From bottom to top on the left: testosterone propionate clearly separated from the two bands of diglycerides. Detection: sulfuric acid.

the blank controls. It also is not suitable for general application as it would be necessary to have available for comparative testing the oil used in the steroid solution.

If our method described here is used instead, the elution time can be extended up to eight hours, along with a proportional increase in the elution volume. It also permits the separation of substances which have small differences in R_F value. This is demonstrated in Fig. 1b where the substances to be separated are identical to those in Fig. 1a; and where the two bands corresponding to the diglycerides are easily identifiable and are clearly separated from each other and from the steroid.

This type of separation is also applicable to oily solutions of steroids other than testosterone propionate as is shown by Figs. 2a and 2b. These figures clearly show the similar behaviour of 19-nortestosterone propionate, testosterone cyclopentylpropionate, progesterone and estradiol benzoate.

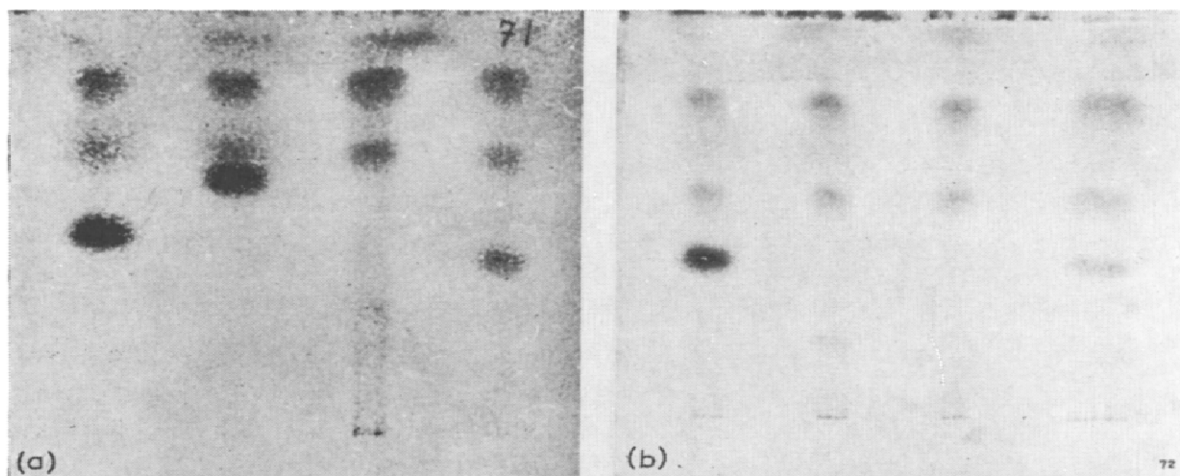

Fig. 2. (a) Chromatogram with solvent 1, continuous development for 8 h. From left to right: testosterone propionate, oily solution (steroid 50 μg, oil 2 mg); testosterone cyclopentylpropionate, oily solution (steroid 50 μg, oil 2 mg); reference oil (2 mg); 19-nortestosterone propionate, oily solution (steroid 20 μg, oil 2 mg). From bottom to top: steroid and the two bands of diglycerides. Detection: sulfuric acid. (b) Chromatogram with solvent 1, continuous development for 8 h. From left to right: testosterone propionate, oily solution (steroid reference); progesterone, oily solution (steroid 20 μg, oil 2 mg); reference oil (2 mg); estradiol benzoate (steroid 10 μg, oil 2 mg). From bottom to top: steroid and the two bands of diglycerides. Detection: sulfuric acid.

This technique of continuous development can be applied also with more polar solvents, reducing the development time proportionally. It was possible, using a petroleum ether b.p. 65°–ethyl ether–acetic acid (50:50:1) mixture for three hours, to obtain excellent separations of both progesterone and testosterone propionate.

Quantitative analysis

The application of this continuous development method to the quantitative analysis of steroids is shown in Tables II and III.

Table II reports the results obtained with pure steroids in alcoholic solution for the purpose of studying their behaviour in the presence of the adsorbent, their recovery, and the determination of their absorptivity along with the standard deviation and the standard error of the mean.

The absorptivity calculated in this manner can then be used to determine the amounts of the various steroids isolated from the oily solutions as shown in

TABLE II

ABSORPTIVITY OF PURE STEROIDS IN ETHANOL SOLUTION

Sample	*Solvent system*	*Quantity of steroid spotted on plate, µg*	*Absorptivity at 240 mµ obtained after elution, a**	*Absorptivity at 380 mµ obtained after elution and reaction with isonicotinic hydrazide, a**
Testosterone propionate, ethanol solution, 5 mg/cm³	1	250	448	328
	1	250	437	338
	1	250	450	327
	1	250	—	326
	1	250	450	357
	1	250	440	326
			445 ± 6.1** ± 2.7***	334 ± 12.3** ± 4.0***
Testosterone propionate, ethanol solution, 5 mg/cm³	2	250	465	—
	2	250	476	—
	2	250	452	—
	2	250	446	—
	2	250	443	—
	2	250	465	—
	2	250	462	—
			458 ± 11.9** ± 4.5***	
Progesterone, ethanol solution, 5 mg/cm³	2	250	526	411
	2	250	526	396
	2	250	527	395
	2	250	524	415
	2	250	536	405
			527 ± 4.8** ± 2.1***	404 ± 8.9** ± 4.0***

* a = absorbance index, absorptivity, ($E^{1\%}_{1\,cm}$).
** Standard deviation.
*** Standard error of the mean.

Table III, as other authors previously mentioned[12–15] have done in the quantitative applications of thin-layer and paper chromatography. The recovery values in these cases were always referred, although with some variations in the different methods, to a standard chromatographed under the same conditions as the sample.

These results allow us to draw the following conclusions. The absorptivity values used as references in the tests with testosterone propionate and progesterone were respectively 491 and 535, in good agreement with those reported in the literature[21] (490 and 540). Absolute recovery ran from 98.5 % for progesterone to 93.3 % for testosterone propionate after a chromatographic development lasting 3 h, and to 90.6 % for the latter steroid after a development lasting 8 h. These results confirm the observation that the behaviour of several steroids may vary with regards to the adsorbent and the chromatographic conditions.

Higher absolute recovery values for progesterone with respect to testosterone

TABLE III

RECOVERY OF STEROIDS IN OILY SOLUTIONS SEPARATED BY THIN-LAYER CHROMATOGRAPHY

Sample	*Solvent system*	*Quantity of steroid spotted on plate*	*Quantity recovered*				*Absorptivity value used in spectrophotometric analysis, a^**	*Absorptivity value used in colorimetric analysis, a^**
			Spectrophotometric		*Colorimetric*			
		µg	*µg*	%	*µg*	%		
Testosterone propionate, oily solution, 25 mg/cm³	1	250	237.5	95.0	238.3	95.3	445	334
	1	250	239.3	95.7	240.8	96.3		
	1	250	241.0	96.4	242.5	97.0		
	1	250	237.0	94.8	239.0	95.6		
	1	250	242.6	96.1	255.3	102.1		
	1	250	241.0	96.4	—	—		
			239.7 ± 2.2**	95.9	243.1 ± 7.0**	97.2		
Testosterone propionate, oily solution, 25 mg/cm³	2	250	248.3	99.3	—	—	458	—
	2	250	243.8	97.5	—	—		
	2	250	251.0	100.4	—	—		
	2	250	243.8	97.5	—	—		
	2	250	243.3	97.3	—	—		
			246.0 ± 3.4**	98.4				
Progesterone, oily solution, 10 mg/cm³	2	250	242.8	97.1	250.5	100.2	527	404
	2	250	242.8	97.1	244.8	97.9		
	2	250	241.3	96.5	244.0	96.7		
	2	250	243.3	97.3	254.6	101.8		
	2	250	247.0	98.8	248.8	99.5		
			243.4 ± 2.1**	97.4	248.5 ± 4.9**	99.4		
Estradiol cyclopentylpropionate, oily solution, 5 mg/cm³	2	250	259.2	103.7	—	—	53.6	—
	2	250	254.0	101.6	—	—		
19-Nortestosterone propionate, oily solution, 10 mg/cm³	2	250	239.1	95.6	—	—	487	—
	2	250	236.0	94.4	—	—		

* a = absorbance index, absorptivity, $(E_{1cm}^{1\%})$.
** Standard deviation.

were observed by MATTHEWS and others[15] under different chromatographic conditions. This confirms additionally the necessity for accurate verification of quantitative thin-layer chromatography under standard working conditions, a criterion which is usual for gas–liquid chromatography.

The results reported in Table III for the experiments done with progesterone and testosterone propionate, and compared with the absorptivity values determined using steroids in ethanol solutions, show excellent recoveries of the same order for the different conditions of chromatography and for the various steroids, *viz.* 97.4, 95.9 and 98.4, all values higher than 95 % in agreement with the findings of ROBERTS AND FLOREY[12].

This technique can be considered of general use for steroids in oily solutions having a Δ^4-3-keto configuration, as is demonstrated by the experiments with 19-nortestosterone propionate. Regarding the estrogens, we limited our tests to estradiol cyclopentylpropionate where the spectrophotometric determinations at the wavelength 282 mμ were possible. In any case the low concentrations of estrogens in oily solutions generally used in pharmaceutical chemistry, and the low absorptivity values in the U.V. of the estrogens cause the measurements to be less precise than for those of the Δ^4-3-keto steroids.

In subsequent tests using oily solutions of estrogens the sulfuric acid–hydroquinone colorimetric method described by BROWN[25] was used to full advantage. This will be referred to in another paper.

It must be pointed out that the satisfactory precision obtainable by this method depends not only on the efficiency of the separation as indicated in Fig. 3, but also on the elution techniques which furnish consistent and low values for the controls.

Absorbance values for the controls averaged 0.024 $\pm$ 0.006 (standard deviation calculated from 17 tests) in comparison to sample absorbance values of 0.445–0.527,

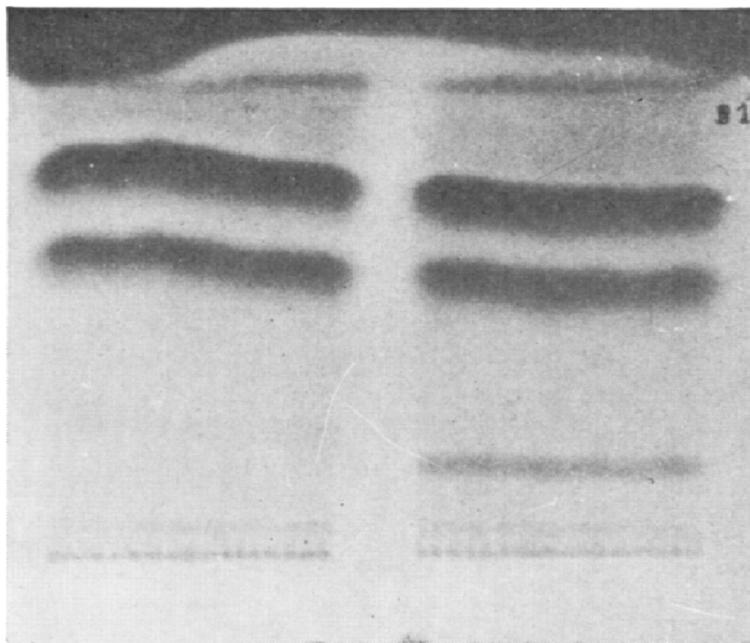

Fig. 3. Chromatogram with solvent 2, continuous development for 3 h. Left: oil 25 mg; right: progesterone 250 μg in 25 mg oil. From bottom to top: progesterone clearly separated from the two diglyceride bands. Detection: iodine vapor.

for equal areas eluted at the same dilution. Performing the elution with ethanol resulted in average extinction values of 0.135 with appreciable differences between various samples. The efficiency of the method with respect to the separation of steroids from fats is shown in Fig. 4, where the absorption spectra of progesterone, the oil control and the adsorbent layer only are reported. The absorbance/wavelength plots for the two types of control are practically coincident, thereby showing the clear separation of the steroid from interfering substances contained in the oil and the possibility of eliminating the control-oil test.

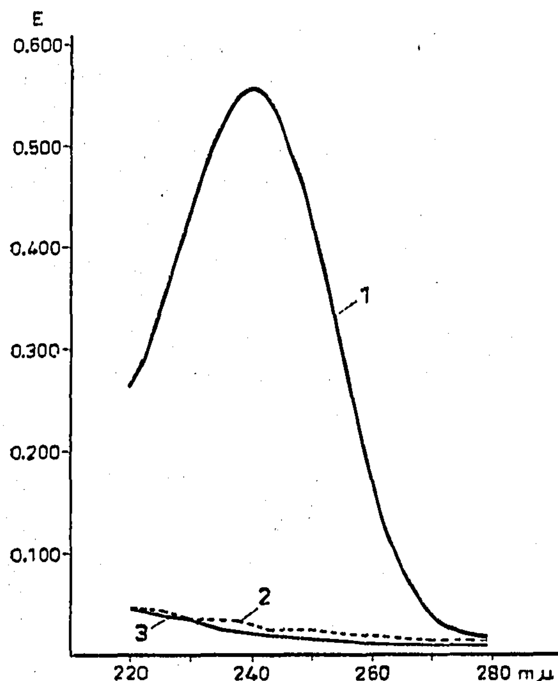

Fig. 4. U.V. absorption spectra of (1) progesterone eluate, (2) reference oil eluate, and (3) adsorbent eluate, from equivalent areas.

In some cases, attempts were made to perform, simultaneously along with the spectrophotometric determinations of the Δ^4-3-keto steroids, determinations with the isonicotinic hydrazide according to the method of UMBERGER[4] which has been widely applied[12–14] in the determination of steroids eluted by paper chromatography. The results obtained were in good agreement with those by the U.V. measurements. Again in this case, the control values were extremely low (oil blank 0.003, adsorbent blank 0.002).

It can be said in conclusion that the simple continuous elution technique developed in our laboratory would possibly give excellent results in the separation of many classes of steroids from lipids. The petroleum ether (b.p. 65°)–ethyl ether–acetic acid mixtures as proposed by MANGOLD[26] for the separation of lipids in classes have wider application to this kind of important separation.

Furthermore this technique gives satisfactory results even in the quantitative analysis of steroids in lipid–steroid mixtures.

SUMMARY

The present paper describes a thin-layer chromatographic method for the determination of some less polar hormonal steroids in oily solution.

By using an ascending technique with continuous elution it is possible to considerably increase the time of the chromatographic run. In this way a ready separation of the steroids is possible, not only from the principal components of the oils (triglycerides) but also from those components (diglycerides and free fatty acids) which under normal conditions of chromatography interfere because of their R_F values.

It is possible to determine testosterone, 19-nortestosterone and estradiol esters and also progesterone spectrophotometrically in the U.V. Other colorimetric determinations may be performed on the same chromatographic eluate.

REFERENCES

1 R. Angelico, G. Cavina, A. D'Antona and G. Giocoli, *J. Chromatog.*, 18 (1965) 57.
2 J. Carol, *J. Assoc. Offic. Agr. Chemists*, 36 (1953) 1000.
3 *Pharmacopoeia of United States*, XV Ed., 1955, p. 717.
4 E. J. Umberger, *Anal. Chem.*, 27 (1955) 768.
5 G. Tappi, E. M. Andreoli and E. Frea, *Pharm. Weekblad*, 93 (1958) 231.
6 D. W. Snair and L. A. Schwinghamer, *J. Pharm. Pharmacol.*, 13 (1961) 148.
7 J. Wolff, *J. Pharm. Sci.*, 52 (1963) 93.
8 H. J. Pazdera, W. H. McMullen, L. L. Ciaccio, S. R. Missan and T. C. Greenfell, *Anal. Chem.*, 29 (1957) 1649.
9 L. M. Reinecke, *Anal. Chem.*, 8 (1956) 1853.
10 G. Cavina, E. Cingolani and A. Giraldez, *Farmaco (Pavia), Ed. Prat.*, 17 (1962) 149.
11 F. M. Kunze, *J. Pharm. Sci.*, 53 (1964) 1170.
12 H. R. Roberts and K. Florey, *J. Pharm. Sci.*, 51 (1962) 794.
13 Drug Standard Laboratory, *J. Pharm. Sci.*, 53 (1964) 98.
14 J. M. Talmage, M. H. Penner and M. Geller, *J. Pharm. Sci.*, 53 (1964) 76.
15 J. S. Matthews, A. L. Pereda and A. Aguilera, *J. Chromatog.*, 9 (1962) 331.
16 G. Cavina and C. Vicari, in G. B. Marini-Bettòlo (Editor), *Thin-layer Chromatography*, Elsevier, Amsterdam, 1064; *Farmaco (Pavia), Ed. Prat.*, 19 (1964) 348.
17 E. P. Schulz, *J. Pharm. Sci.*, 54 (1965) 146.
18 H. G. Ganshirt and J. Polderman, *J. Chromatog.*, 16 (1964) 510.
19 H. L. Bird, Jr., H. F. Brickley, J. P. Comer, E. P. Hartsaw and M. L. Johnson, *Anal. Chem.*, 35 (1963) 346.
20 B. P. Korzun and S. Brody, *J. Pharm. Sci.*, 52 (1963) 206.
21 *British Pharmacopoeia*, 1963, pp. 16 and 666.
22 *National Formulary*, XI Ed., Am. Pharm. Assoc., 1960, p. 131.
23 E. Cingolani, G. Cavina and V. Amormino, *Farmaco (Pavia), Ed. Prat.* 15, (1960) 301.
24 G. Cavina, E. Cingolani, V. Amormino and A. Giraldez, *Farmaco! (Pavia), Ed. Prat.*, 17 (1962) 393.
25 J. B. Brown, *Biochem. J.*, 60 (1955) 185.
26 H. K. Mangold, *J. Am. Oil Chemists' Soc.*, 38 (1961) 708.