CHROM. 3928

GAS CHROMATOGRAPHIC AND COLORIMETRIC DETERMINATION OF ESTRADIOL MONOESTERS IN OIL SOLUTIONS AFTER SEPARATION BY THIN-LAYER CHROMATOGRAPHY

G. MORETTI, G. CAVINA AND J. SARDI DE VALVERDE*
Istituto Superiore di Sanità, Rome (Italy)

(Received November 23rd, 1968)

SUMMARY

A method is described for the determination of estradiol monoesters in oil solutions, for pharmaceutical use (concentration 2 mg/ml). The separation of the steroid is performed by means of thin-layer chromatography followed by a colorimetric or a gas chromatographic method for quantitative analysis. As examples, the procedure for the analysis of estradiol-17β-cyclopentylpropionate (1) and estradiol-3-benzoate (2) is reported. The TLC step is similar in both cases; in example (1) a saponification technique is applied to the steroid ester adsorbed on the silica gel thus achieving simultaneous elution and hydrolysis to estradiol, which is then extracted with ethyl ether. In example (2) a simple elution with chloroform is performed. The estradiol (example 1) or its monoester (example 2) is transformed into the respective trimethylsilyl ether (TMSE) prior to gas chromatography. For this step a method previously described by the authors for diesters of estradiol or estrone esters is employed. The colorimetric analysis is performed by BROWN's method[2] as described for urinary estrogens, with the correction of the absorption values according to ALLEN[3]. Recovery values and the precision of both methods are reported.

INTRODUCTION

In a previous paper[1] we described the separation of some derivatives of estrone and estradiol in oil solutions, for pharmaceutical use, at a concentration of 2 mg/ml by means of thin-layer chromatography (TLC).

This separation, verified quantitatively for solutions of estrone-3-benzoate and estradiol-3,17-dipropionate, allows the determination of the estrogens by two different methods; *viz.*, a colorimetric one using the reaction of sulfuric acid and hydroquinone according to BROWN[2], with correction of the spectrophotometric readings according to ALLEN[3], and a gas chromatographic (GLC) one described by us. The latter, an accurate and sensitive method, is convenient for laboratories equipped for gas chromatographic analysis, being more rapid and specific for all estrogens tested so

* Fellow of the Laboratory of Biology.

far, than the colorimetric one, has recently been studied by us with the aim of making its application more general.

In our previous work we studied completely esterified estrogens, which can be directly analyzed by GLC after separation by TLC, because of their stability in the working conditions. In this paper two different separation methods with different, simple elution or elution–saponification steps, and subsequent protection of the hydroxyl are described for two monoesters of estradiol (in the 3 and 17 position), estradiol-3-benzoate and estradiol-17β-cyclopentyl propionate. These methods can also be applied to other similar esters. In particular, the elution–saponification method permits the analysis of different esters of estradiol by the same gas chromatographic method.

EXPERIMENTAL

Reagents

Ethyl ether and ethanol were purified as described by us previously[1]; hexane (benzine for chromatography b.p. 65°), methanol, chloroform, carbon disulfide were of analytical grade (a.g.). Sulfuric acid, acetic acid, hydroquinone and benzyl alcohol were also a.g.; olive oil for pharmaceutical use was refined and deacidified. Materials for TLC were Merck Silica Gel G, washed for 24 h with chloroform a.g. in a Soxhlet; this material was also used in some cases mixed with 0.3% Dupont Luminescent Chemical 609.

Reference compounds: Estradiol-17β, estradiol-3-benzoate, estradiol-17β-cyclopentylpropionate, 5α-cholestane, 5α-cholestane-3β-ol propionate (cholesteryl propionate) were analytical grade and if necessary recrystallized. Materials for gas chromatography were 80–100 mesh Gas-Chrom Q and SE-30 (methylsiloxane polymer).

Preparation of the solutions for analysis

(a) Reference solutions. The steroids to be analyzed were dissolved in ethanol at a concentration of 0.8 mg/ml and diluted to 5 and 8 μg/ml as necessary.

(b) Solutions in oil. These were prepared in a tared 10 ml glass stoppered cylinder by dissolving 8 mg of steroid in 0.1 ml of benzyl alcohol and adding olive oil to a volume of 4 ml; this solution of steroid in oil (concentration 2 mg/ml) was diluted with heptane to a final volume of 10 ml (concentration of the steroid 0.8 mg/ml, concentration of oil 0.4 ml/ml: solution b_1).

(c) Blank (oil only). A solution of oil in heptane at a concentration of 0.4 ml/ml was prepared.

(d) Standard solutions. Internal standards for GLC were prepared in heptane.

Procedure for the isolation and determination of steroids

(1) Estradiol-17β-cyclopentylpropionate

(A) Chromatographic separation. A thin-layer separation on Silica Gel G plates was performed, thickness of layer 0.5 mm, solvent hexane–ethyl ether–acetic acid (HEAA) (80:20:1), with continuous development for 3 h according to the technique described by CAVINA AND MORETTI[4]. Of the solution in heptane b_1 50 μl (40 μg steroid andapproximately 20 mg oil), were applied with a Hamilton microsyringe in a line

of 6 cm length. The steroid was identified by spraying a central lane (on which 8 μl of the same solution b_1 had been applied) with Folin-Ciocalteu's reagent (1:5) (ref. 5) and with 4 *N* NaOH. For details of the chromatographic analysis see the preceding papers[1,4].

(B) Preparation of the derivative for gas chromatographic analysis. After identification of the steroid band the corresponding silica gel (approximately 2 × 7.5 cm) was scraped off and transferred into a 30 ml glass stoppered test tube and the saponification of the estrogen ester was carried out with 4 ml 10% KOH (w/v) in methanol, by refluxing in a boiling water bath for 30 min. After cooling, 3 ml of 2 *N* HCl, 3 ml of water and 6 ml of purified and freshly distilled ethyl ether were added to the tube. After shaking, the ether phase was sucked off with a pipet and transferred into a 25 ml separator equipped with a teflon stopcock. Four successive extractions each with 2 ml ether were carried out. The collected ether extracts were washed with 2 ml of 2.5% (w/v) $NaHCO_3$ and twice with 2 ml water. The ether phase was dehydrated by passage through anhydrous sodium sulfate and filtered into a 25 ml volumetric flask. The sample was brought to dryness by evaporating the ether under a stream of nitrogen, 0.5 ml methanol was added and made up to volume with chloroform (solution C_p). Of the solution C_p 5 ml were transferred into a glass stoppered test tube and taken to dryness; 1 ml (8 μg) of the standard solution (d) of 5α-cholestane was added to the residue, the solvent was again evaporated and the residue was left overnight under vacuum in a desiccator over KOH and silica gel. In order to prepare the trimethylsilyl ethers (TMSE) according to BOUGHTON *et al.*[6], 0.1 ml of the appropriate reagent (anhydrous pyridine, hexamethyldisilazane and trimethylchlorosilane, 9:3:1) was added to the dried residue. The tube was well stoppered and left for 1 h at room temperature, then the sample was evaporated to dryness under a stream of dry nitrogen, the walls of the tube were washed with a small amount of carbon disulfide, the solution was again evaporated and the residue was dissolved in the tip of the tube in 40 μl of the same solvent. For each analysis a quantity of 2 μl was injected into the gas chromatograph.

(C) Gas chromatographic analysis. The instrument was a Perkin-Elmer model 801 gas chromatograph equipped with a flame ionization detector and a 5 mV recorder. A 2.20 m helical glass column, I.D. 2.5 mm, packed with 2% SE-30 on 80–100 mesh Gas-Chrom Q was used. The column was preconditioned as described earlier[1]. The temperatures of the oven, injector and detector were 190°, 260° and 200°, respectively. The nitrogen flow rate was 40 ml/min. The attenuation was set at × 5. For the calibration curve aliquots of 2.5, 5.0 and 7.5 μg of estradiol-17β standard were transferred, in duplicate, into conical tubes, 8 μg (1 ml) of 5α-cholestane (internal standard) were added and the samples were evaporated to dryness. The trimethylsilylethers were prepared as described before for the sample under (B). Their value $R = A/B \cdot C/D$ was calculated according to CELESTE AND TURCZAN[7] where A = peak height of analyzed steroid, B = peak height of internal standard, C = μg of internal standard and D = μg of analyzed steroid. The average R value determined was ($m \pm$ S.D.) 1.620 ± 0.006. The estradiol-17β-cyclopentylpropionate value was calculated by multiplying the estradiol found by 1.46.

(D) Colorimetric analysis. After evaporation to dryness of 6.5 ml of the solution C_p, the reaction with sulfuric acid (60% v/v) and hydroquinone as described by BROWN[2], and the correction of the absorption values according to ALLEN[3], was

used for the determination. The measurements were carried out on a Beckman DU-2 spectrophotometer at the wavelengths of 476, 514 and 552 nm.

The calibration curve was prepared with estradiol-17β; the value found was calculated for estradiol-17β-cyclopentylpropionate using the factor mentioned above.

(2) Estradiol-3-benzoate

(A) Chromatographic separation. The procedure was analogous to that described for estradiol-17β-cyclopentylpropionate. The solvent was hexane–ethyl ether–acetic acid (50:50:1) with continuous development for 1 h and 30 min.

The identification of the compound examined was performed under U.V. light (0.3% Dupont Luminescent Chemical 609 was incorporated in the silica gel).

(B) Preparation of the derivative for gas chromatographic analysis. After identification of the steroid band, the corresponding silica gel (approximately 2 × 7.5 cm) was transferred into a small tube, 0.8 cm in diameter, plugged at the bottom with a small layer of defatted cotton, and was eluted with 25 ml of chloroform (solution C_p). The preparation of the 17β-trimethylsilyl ether of estradiol-3-benzoate was performed with 5 ml of solution C_p which had been taken to dryness in a glass stoppered conical tube. The procedure was the same as in the example described above. A solution of cholesteryl propionate in heptane at a concentration of 5 μg per 1 ml was used as internal standard.

(C) Gas chromatographic analysis. The instrument and column were the same as described for estradiol-17β-cyclopentylpropionate analysis. The temperatures of the oven, injector and detector were 235°, 260° and 235° respectively; the nitrogen flow rate was 40 ml/min; the attenuator was set at × 5. For the calibration curve aliquots of 4, 8, 12 μg of estradiol-3-benzoate were used, in duplicate, and 5 μg (1 ml) of cholesteryl propionate were added. The trimethylsilyl ethers were prepared and their R value was determined as described above. The average R value was 0.683 ± 0.017.

(D) Colorimetric analysis. The procedure was the same as described before for estradiol-17β-cyclopentylpropionate. The measurements were carried out at the wavelengths 480, 516 and 552 nm; the calibration curve was prepared with estradiol-3-benzoate.

The procedures of chromatographic separation and gas chromatographic analysis, including the preparation of the derivative, were also performed with the solution (c), the blank of oil only.

RESULTS AND DISCUSSION

Figs. 1 and 2 are photographs of thin-layer chromatograms which show the separation of the estrogens studied, estradiol-17β-cyclopentylpropionate and estradiol-3-benzoate, from the oil. The separation is better for the latter steroid: the conditions used are the result of several experiments carried out with solvent systems already described by us[1].

The results of analysis of solutions with a known quantity of the estrogens studied are presented in Table I. The data obtained show the good reproducibility and accuracy of the procedures studied. As already shown in our previous work[1] concerning the colorimetric analysis, the results obtained reaffirm the applicability of the method described for different derivatives of estrone and estradiol.

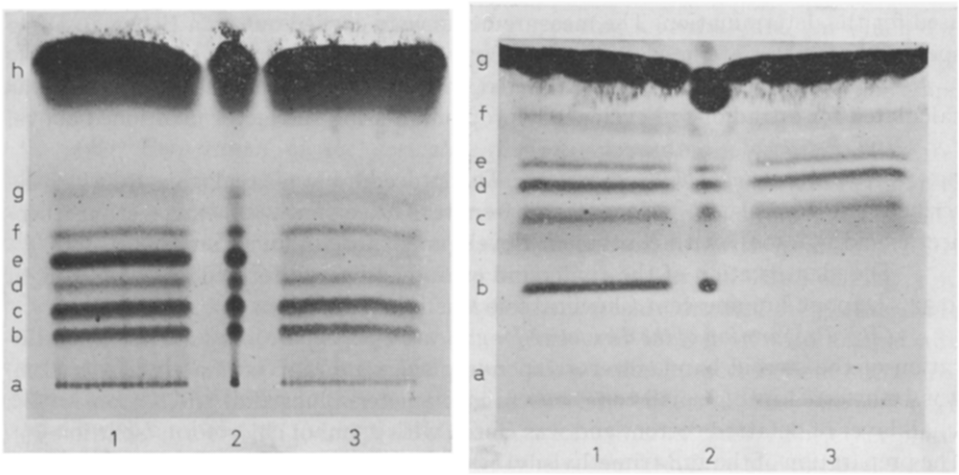

Fig. 1. TLC with HEAA (80:20:1), continuous development for 3 h. (1) Estradiol-17β-cyclopentylpropionate in oil solution; (2) *idem*, reference lane; (3) reference oil blank. (a) Monoglycerides (traces); (b) 1,2-diglycerides; (c) 1,3-diglycerides; (d) and (f) minor components (not identified); (e) estradiol-17β-cyclopentylpropionate; (g) free fatty acids; (h) triglycerides. Detection: 50% H_2SO_4 and heating.

Fig. 2. TLC with HEAA (50:50:1), continuous development for 1.5 h. (1) Estradiol-3-benzoate in oil solution; (2) *idem*, reference lane; (3) reference oil blank. (a) Monoglycerides (traces); (b) estradiol-3-benzoate; (c) 1,2-di-glycerides; (d) 1,3-diglycerides; (e) and (f) minor components (not identified); (g) triglycerides. Detection: 50% H_2SO_4 and heating.

It should be noted here that the procedures of elution and preparation of derivatives described for estradiol-17β-cyclopentylpropionate and estradiol-3-benzoate are applicable for the gas chromatographic analysis of all the derivatives of estradiol.

We think the elution–saponification procedure may be of particular interest as it can be considered as generally applicable to esters of estradiol which can be analyzed as estradiol diTMSE, after saponification; in all cases the same gas chromatographic technique was used.

TABLE I

RECOVERY OBTAINED BY GAS CHROMATOGRAPHIC AND COLORIMETRIC ANALYSIS

The number of determinations is indicated in brackets.

Steroid	*Quantity of steroid used for the analysis (µg)*	*Analytical procedure for separation and gas chromatography*	*Recovery by gas chromatographic method*		*Recovery by colorimetric method (absorbancies are corrected with* ALLEN*'s formula)*	
			Mean ± S.D.	*Percentage*	*Mean ± S.D.*	*Percentage*
Estradiol-17β-cyclopentylpropionate in oil solution (2 mg/ml)	40.0	1 (6)	39.7 ± 1.1	99.2 ± 2.7	38.9 ± 1.1	97.2 ± 2.7
Estradiol-3-benzoate in in oil solution (2 mg/ml)	40.0	2 (5)	40.0 ± 1.3	100.0 ± 3.2	40.5 ± 1.0	101.2 ± 2.5

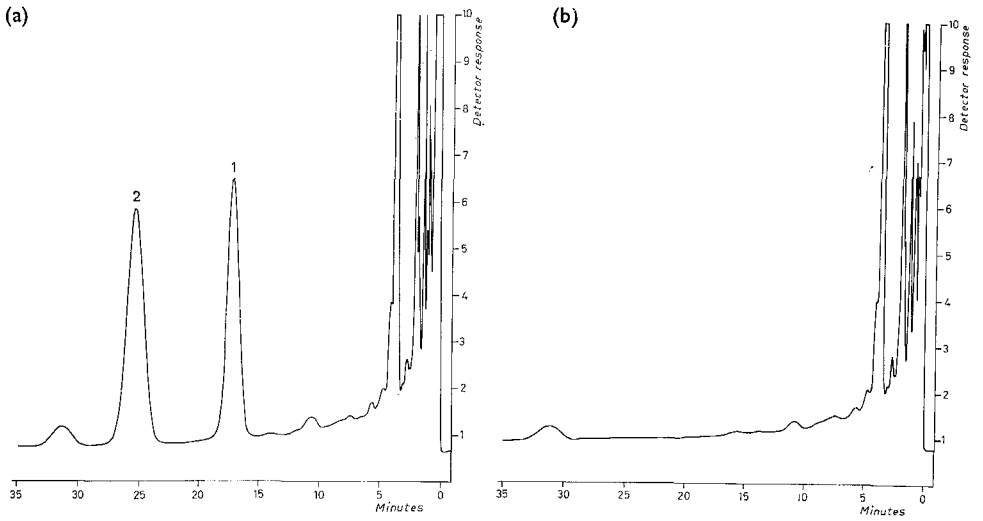

Fig. 3. (a) Gas chromatogram of the eluate of an oil solution of estradiol-17β-cyclopentylpropionate after chromatography, saponification to estradiol, and TMSE preparation. Peak 1: 5α-cholestane. Peak 2: estradiol diTMSE. Column: 2% SE-30 on 80–100 mesh Gas-Chrom Q, 2.20 m length, 190°. (b) Gas chromatogram of the reference oil blank subjected to the same procedure.

The conditions of saponification have been studied in a number of experiments under different conditions, with acids and bases, in water and alcohol media and for different reaction times. The best reaction conditions were those described, giving the highest percentage recovery for the saponified product (also quantitatively controlled by TLC) and assuring the best conditions for the stability of the free estradiol. The saponification procedure and successive gas chromatographic and colorimetric analyses were verified on 40 μg pure estradiol-17β-cyclopentyl propionate*, the following results being obtained: 39.7 $\pm$ 0.8 μg (99.3 $\pm$ 1.9%) by the gas chromatographic method, and 39.9 $\pm$ 1.3 μg (99.9 $\pm$ 3.3%) by the colorimetric one with the correction according to ALLEN[3] (average values of 5 experiments $\pm$ S.D.). Experiments with estradiol gave values of the same order of magnitude.

Our results are comparable with and are, for estrogen alone, better than those obtained by BILLIAR AND EIK-NES[8] after saponification with cholinesterase and sodium carbonate of different steroid acetates, including estradiol. The better results obtained by us with alkali in the presence of silica gel are probably due to the buffering effect of the latter; this is supported by the observations of HORNSTEIN *et al.*[9] on the saponification of lipids under conditions similar to those of our experiments.

The simultaneous elution and saponification procedure can be advantageous not only for the gas chromatographic analysis but also for the colorimetric one: For example, in the case of estradiol-17β-cyclopentylpropionate the direct determination of the estrogen in the chloroform eluate from the silica gel was upset by interfering substances which considerably lowered the recovery and caused its inconstancy. This interference disappeared when performing the analysis with the saponification prod-

* The silica gel blank was obtained from an area, 7.5 $\times$ 2 cm, of a layer developed without a sample.

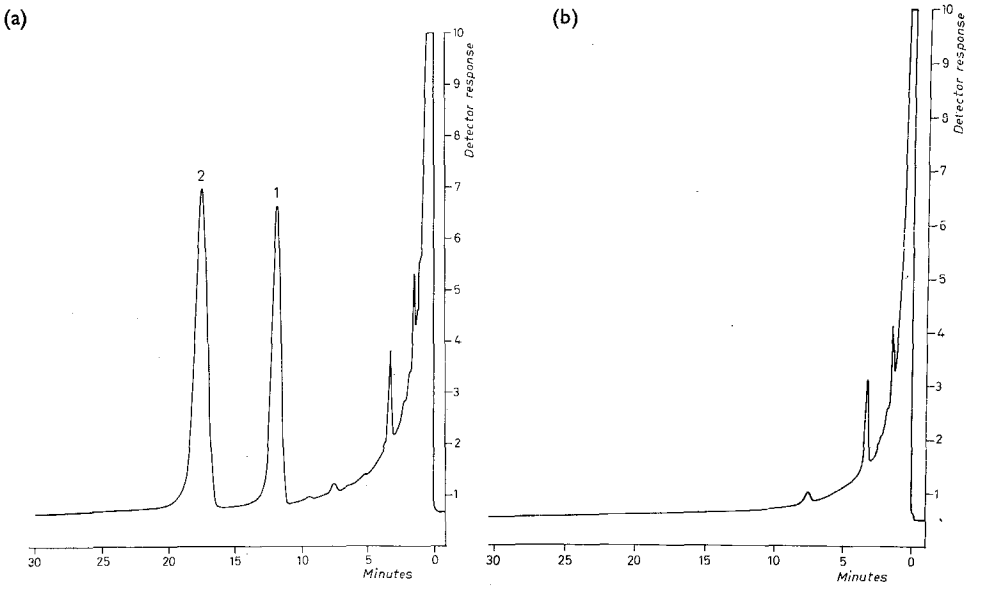

Fig. 4. (a) Gas chromatogram of the eluate of an oil solution of estradiol-3-benzoate after chromatography. Peak 1: cholesteryl propionate. Peak 2: estradiol-3-benzoate-17-TMSE. Column: 2% SE-30 on 80–100 mesh Gas-Chrom Q, 2.20 m length, 235°. (b) Gas chromatogram of the reference oil blank subjected to the same procedure.

uct. In Fig. 1 the complexity of the chromatographic separation is clearly shown. As the concentration of the estrogen is only 0.2% w/v in the oil solution, every component of the oil present with this order of concentration and, with an R_F value near to that of the substance to be separated, can cause interference.

The possibility of choosing convenient techniques of separation, elution and determination also permits one to overcome those difficulties which may be encountered with different substances in different ways.

The second procedure is simpler in its application and allows a more rapid gas chromatographic analysis of the monoesters of estradiol, when they can be eluted without interference from thin-layer chromatograms.

Figs. 3(a) and (b) and 4(a) and (b) show the gas chromatograms of the estrogens studied and the respective oil blanks. They show the good separation of the peaks of the steroids and of the internal standards from some other low peaks which are due to unknown components in the oil.

REFERENCES

1 G. CAVINA, G. MORETTI AND J. SARDI DE VALVERDE, *Ann. Ist. Super. Sanita*, 4 (1968) 75.
2 J. B. BROWN, *Biochem. J.*, 60 (1955) 185.
3 W. M. ALLEN, *J. Clin. Endocrinol. Metab.*, 10 (1950) 71.
4 G. CAVINA AND G. MORETTI, *J. Chromatog.*, 22 (1966) 41.
5 F. L. MITCHELL AND R. E. DAVIES, *Biochem. J.*, 56 (1954) 690.
6 O. D. BOUGHTON, R. BRYANT, W. J. LUDWIG AND D. L. TIMMA, *J. Pharm. Sci.*, 55 (1966) 951.
7 A. CELESTE AND J. TURCZAN, *J. Assoc. Offic. Agr. Chemists*, 46 (1963) 1055.
8 R. B. BILLIAR AND K. B. EIK-NES, *Anal. Biochem.*, 13 (1965) 11.
9 I. HORNSTEIN, P. CROWE AND B. RUCK, *J. Chromatog.*, 27 (1967) 485.