obtained, m.p. 146–147° from 95% ethanol. [Lit. (1) 145–147.3°.] $\nu_{max.}$ 3048, 3022 (aromatic C—H); 1620 (C=O). $\lambda_{max.}$ 248 (ϵ 32,000); 301 (ϵ 6,800); 387 (ϵ 11,700). The ultraviolet absorption maxima did not undergo appreciable wavelength shifts in 4 *N* hydrochloric acid in 50% ethanol.

REFERENCES

(1) Harfenist, M., *J. Org. Chem.*, **28**, 1834(1963).
(2) Bellamy, L. J., "Infra-red Spectra of Complex Molecules," 2nd. ed., John Wiley & Sons, Inc., New York, N. Y., 1958, pp. 259, 260.
(3) Van Allan, J. A., Reynolds, G. A., and Adel, R. E., *J. Org. Chem.*, **27**, 1659(1962).
(4) Hromatka, O., Knollmüller, M., and Sauter, F., *Monatsh. Chem.*, **93**, 723(1962).
(5) Schmalz, A. C., and Burger, A., *J. Am. Chem. Soc.*, **76**, 5455(1954).
(6) Babcock, S. H., and Fuson, R. C., *ibid.*, **55**, 2946 (1933).
(7) Fischer, O., and Chur, M., *J. Pract. Chem.*, **93**, 363 (1916).
(8) Hebky, J., and Radek, O., *Chem. Listy*, **46**, 637(1952).

Keyphrases

Tetracyclic phenothiazines
Bromination of pyrido[3,2,1-*kl*] phenothiazines
IR Spectrophotometry–structure
UV Spectrophotometry–structure

Quantitative Separation and Estimation of Steroid Mixtures by Thin-Layer Chromatography II. Determination of Progesterone and Estradiol Benzoate and of Progesterone, Testosterone Propionate, and Estradiol Benzoate in Mixtures

By TATJANA BIĆAN-FIŠTER

Rapid and simple procedures were developed for the TLC separation and subsequent quantitative determination of progesterone and estradiol benzoate as well as progesterone, testosterone propionate, and estradiol benzoate in vegetable oil solutions and microcrystalline water suspensions. The solvent system proposed permits not only a rapid quantitative separation of the steroids from one another but at the same time enables the separation of the steroids from the vegetable oil. No special extraction of steroids from the oil is thus necessary. The separated steroids after extraction from the adsorbent are each determined by the colorimetric and fluorimetric method, respectively.

In a recent communication (1) concerning the quantification on a microscale of steroid mixtures after thin-layer chromatographic (TLC) separation a procedure was proposed for the determination of progesterone and testosterone propionate in oil solution.

Because of the lack of rapid methods for the routine control of preparations containing steroid mixtures, the present communication describes rapid and simple procedures for the determination of mixtures of progesterone and estradiol benzoate as well as progesterone, testosterone propionate or butyrate, and estradiol benzoate.

The procedures described have been adapted to the quantification of steroid mixtures in vegetable oil solutions and crystalline water suspensions commercially available in Yugoslavia.

EXPERIMENTAL

Materials and Apparatus

Reagents—Dissolve 0.4 Gm. of isonicotinhydrazide in methanol, add 0.5 ml. hydrochloric acid (37%), and fill up to 100 ml. with methanol. Set aside the reagent for 24 hr. before use.

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Adsorbent—Fluorescent Kieselgel HF_{254} (E. Merck, Darmstadt).

Reference Standards—Progesterone, testosterone propionate, estradiol benzoate [Organon, Oss (Holland)].

Apparatus—Thin-layer chromatography outfit with regulation thickness spreader (Desaga, Heidelberg), Agla micrometer syringe (Burroughs Wellcome & Co., London), ultraviolet lamp, 254 mμ (Hanau), fluorimeter [Kipp (Delft)].

Solvent System—Cyclohexane–ether–ammonia (8:2:0.5 v/v).

Preparation of Plates

Chromatoplates were prepared following the technique described by Stahl. Plates 20 × 20 cm. were coated (0.5 mm. layer thickness) with a slurry prepared by mixing with a pestle 35 Gm. Kieselgel HF_{254} with 85 ml. of water in a mortar. A batch suffices for six plates. The plates were air-dried for 10 min. at room temperature and thereafter activated by heating at 130° for 4 hr. and stored in a desiccator until used.

Procedures

A—TLC Separation of Progesterone and Estradiol Benzoate in Oil Injection Solutions—A 3.0-ml.

quantity of the olive oil solution (about 60 mg. progesterone and about 9.0 mg. estradiol benzoate) was diluted in a 10-ml. volumetric flask with chloroform up to the mark. Twice, 20 μl. of this sample solution (samples *1* and *2*) and 20 μl. of each of the corresponding standard solutions were applied with a microsyringe on a Kieselgel HF $_{254}$ coated plate along the starting line (2 cm. from the lower edge) as 2 cm. horizontal lines. The chromatogram was developed in a chamber previously saturated for 24 hr. with the solvent. When the solvent reached about 1 cm. from the upper edge of the plate (about 60 min.), the plate was removed from the chamber, air-dried for a few minutes, and the separated steroids located under low wavelength light (Fig. 1). The UV absorbing zones of the sample and the standards were marked with an ample margin around each zone and quantitatively scraped off the plate, each into a separate 50-ml. glass-stoppered flask. Progesterone was thus extracted from the adsorbent with 5 ml. methanol while estradiol benzoate was extracted with 5 ml. chloroform. The flasks were stirred well for 30 min. and centrifuged.

Determination of Progesterone—Three-milliliter aliquots of each supernatant solution of sample and standard progesterone were pipeted into 50-ml. flasks, 4 ml. of the INH reagent was added, and the mixtures were set aside for 1 hr. The absorbances were measured at 380 mμ against a reagent blank.

$$\text{concn.}_x = \frac{\text{concn.}_{\text{st.}} \cdot A_x}{A_{\text{st.}} \cdot 3}$$

concn.$_x$ = concentration of progesterone per ml.,
concn.$_{st.}$ = concentration of progesterone in 10 ml. standard solution,
A_x = mean value of absorptivities of samples *1* and *2*,
A_s = absorptivity of standard solution,
3 = dilution factor.

Determination of Estradiol Benzoate—Three-milliliter portions of each supernatant solution of sample and standard estradiol benzoate were evaporated to dryness in 10-ml. volumetric flasks by heating on a water bath. The residues were dissolved in 88% sulfuric acid filled to the mark with the same acid. After 1 hr. the developed fluorescences were determined relative to the standard fluorescent solution of estradiol benzoate using filter B_2.

$$\text{concn.}_x = \frac{\text{concn.}_{\text{st.}} \cdot A_x}{A_{\text{st.}} \cdot 3}$$

concn.$_x$ = concentration of estradiol benzoate per ml.,
concn.$_{st.}$ = concentration of estradiol benzoate in 10 ml. standard solution,
A_x = mean value of absorptivities of samples *1* and *2*,
A_s = absorptivity of standard solution,
3 = dilution factor.

Standard Solution of Progesterone—Dissolve 60.0 mg. progesterone in a 10-ml. volumetric flask in chloroform and fill to the mark with the same solvent.

Standard Solution of Estradiol Benzoate—Dissolve 9.0 mg. estradiol benzoate in a 10-ml. volumetric flask in chloroform and fill to the mark with chloroform.

Standard Fluorescent Solution of Estradiol Benzoate—Dilute 10 μl. of the standard solution of estradiol benzoate in a 10-ml. volumetric flask with 88% sulfuric acid to the mark.

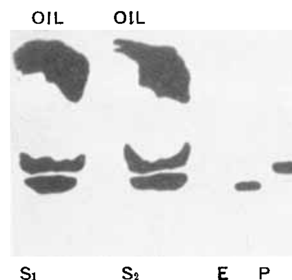

Fig. 1—Chromatogram for olive oil solution of progesterone and estradiol benzoate solution. Key: S_1, S_2 = *duplicate test samples;* E = *estradiol benzoate;* P = *progesterone.*

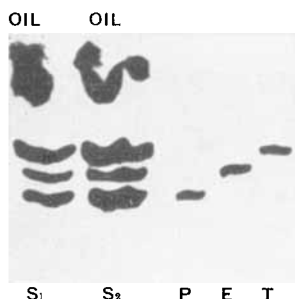

Fig. 2—Chromatogram for olive oil solution of progesterone, estradiol benzoate, and testosterone propionate. Key: S_1, S_2 = *duplicate test samples;* P = *progesterone;* E = *estradiol benzoate;* T = *testosterone propionate.*

B—TLC Separation of Progesterone, Testosterone Propionate, and Estradiol Benzoate in Oil Injection Solution—A 3.0-ml. quantity of the olive oil solution (about 60 mg. progesterone, 75 mg. testosterone propionate, and 9 mg. estradiol benzoate) was diluted in a 10-ml. volumetric flask filled to the mark with chloroform. Proceed as described under *TLC Separation of Progesterone and Estradiol Benzoate* in procedure *A* using the additional standard solution of testosterone propionate (Fig. 2).

Determination of Progesterone and Testosterone Propionate—Three-milliliter aliquots of each supernatant solution of sample and standard progesterone and testosterone propionate were pipeted into 50-ml. volumetric flasks and the determination of both steroids proceeded as described under *Determination of Progesterone* in procedure *A*.

Determination of Estradiol Benzoate—A 3.0-ml. quantity of each standard and sample supernatant chloroform solution of estradiol benzoate was treated in the same manner as described under *Determination of Estradiol Benzoate* in procedure *A*.

Standard Solution of Testosterone Propionate—Dissolve 75.0 mg. testosterone propionate in a 10-ml. volumetric flask in chloroform and fill to the mark with chloroform.

C—Testosterone Butyrate and Estradiol Benzoate in Microcrystalline Water Suspension—The contents of 1 ampul (about 7.5 mg. estradiol benzoate and 150 mg. testosterone propionate) is extracted in a separator with three times 10 ml. chloroform. The combined chloroform extracts are collected in a 50-ml. volumetric flask which is then filled to the mark with chloroform. Twenty microliters of this solution and of the corresponding standard solutions are applied to a Kieselgel HF_{254} plate along the start as 2-cm. horizontal lines, and the separation and determination of testosterone butyrate is carried out as

directed for progesterone, and that of estradiol benzoate as directed for the same steroid in procedure *A*.

Standard Solution for Testosterone Butyrate—Dissolve 150.0 mg. testosterone butyrate in a 10-ml. volumetric flask in chloroform and fill to the mark with chloroform.

Standard Solution of Estradiol Benzoate—Dissolve 10.0 mg. estradiol benzoate in a 10-ml. volumetric flask in chloroform and fill to the mark with the solvent.

D—Progesterone and Estradiol Benzoate in Microcrystalline Suspension—The contents of 1 ampul (about 200 mg. progesterone and 10 mg. estradiol benzoate) are extracted in the same manner as described under procedure *C*. Twice, 20 μl. of the chloroform extract and 20 μl. each of the corresponding standard solutions are applied to the plate, and the separation and determination of steroids are carried out as directed under procedure *A*.

Standard Solution of Progesterone—Dissolve 40.0 mg. progesterone in a 10-ml. volumetric flask in chloroform and fill up to the mark with chloroform.

Standard Solution of Estradiol Benzoate—Dissolve 10.0 mg. estradiol benzoate in a 10-ml. volumetric flask in chloroform and fill up to the mark with chloroform.

RESULTS AND DISCUSSION

The separation of progesterone from estradiol benzoate and progesterone, testosterone propionate, and estradiol benzoate achieved best resolution when 0.5 ml. of ammonia was added to the nonpolar cyclohexane–ether system to make it more polar. [The nonpolar cyclohexane–ether system was previously used (1) for the separation of progesterone and testosterone propionate.] It is known that the R_f values of steroids mainly depend on the number of C and O atoms, *i.e.*, the C/O index, which is largely determined by the polarity of the molecule. The migration of steroids on the plate can thus be explained by the fact that the less polar ester, testosterone propionate, with a longer side chain and a C/O index of 7.3, migrated faster than the more polar diketone, progesterone, with a C/O index of 10.3, and the phenolic ester, estradiol benzoate, with a C/O index of 8.3 (Fig. 2).

The methods used for the quantification of steroids extracted from the adsorbent permit the determination of about 50 mcg. of testosterone propionate and progesterone and about 6 mcg. of estradiol benzoate. This is important since steroids are generally compounded in pharmaceutical formulations in low concentrations. For the determination of the Δ^4-3 ketosteroids, progesterone and testosterone propionate, the colorimetric method by Umberger (2) was adapted. The visual fluorescence of estradiol benzoate, in sulfuric acid solution as an approach to determining steroid substances present in preparations in submicrogram amounts was found to be very useful. Data given by Szalikowsky (3) on the sulfuric acid fluorescence of various steroids proved valuable as a basis for the development of a quantitative procedure. It was found that steroid levels of 3 mg./ml. in 88% sulfuric acid could be measured satisfactorily. The Beer-Lambert law was obeyed over a suitable range of 20 to 140 mcg. of the steroid.

TABLE I—ANALYSIS OF STANDARD STEROID MIXTURES

	Progesterone	Estradiol Benz.	Progesterone	Testosterone Prop.	Estradiol Benz.
	Added, mcg.		Added, mcg.		
	72.00	7.20	72.00	9.00	10.00
No.	Recovered, mcg.		Recovered, mcg.		
1	71.00	7.34	70.71	9.00	10.28
2	71.62	6.81	71.79	8.77	9.82
3	71.79	7.38	70.80	8.92	10.19
4	72.37	7.28	72.92	8.65	9.69
5	72.52	7.01	72.19	8.98	11.09
6	71.44	7.35	71.67	9.08	9.98
7	72.00	7.27			
8	71.12	7.12			
9	71.12	6.91			
Mean	71.67	7.18	71.68	8.90	10.17
Limits of error ($P = 0.95$)	99.40–100.60	96.37–103.63	96.60–103.40	97.10–102.90	98.04–101.96

TABLE II—ANALYSIS OF COMMERCIAL MULTIPLE PHARMACEUTICAL PREPARATIONS

Prepn.	Label Claim	Found, mg.		% of Label Claim	
Oil Injection Soln.					
Progesterone	20.0	19.2	20.1	96.1	100.5
Estradiol benz.	2.0	1.8	2.0	94.4	100.0
Progesterone	20.0	20.4	20.8	102.0	104.0
Testosterone prop.	25.0	23.6	25.8	94.3	103.2
Estradiol benz.	3.0	2.8	3.2	93.6	106.6
Microcrystalline Water Suspension					
Testosterone butyr.	50.0	47.9	49.8	95.8	98.0
Estradiol benz.	2.5	2.5	2.6	100.0	104.0
Progesterone	200.0	193.0	195.8	96.5	97.9
Estradiol benz.	10.0	9.9	10.5	99.6	105.2

The effect of time on the formation of fluorescence was determined by treatment of 20 mcg. of estradiol benzoate with 10 ml. of 88% sulfuric acid. The fluorescence was stabilized at room temperature after 1 hr. and was found to be practically stable for an additional hour.

To determine the precision of the procedures, analyses were carried out with standard steroid vegetable oil solutions prepared in this laboratory and containing exactly known quantities of steroids in approximately the same concentrations present in commercial preparations.

It is evident from the data in Table I that good reproducibility with a satisfactory standard deviation is obtained. The determination of steroids in commercial dosage forms showed that the results obtained are in good agreement with the labeled amount of the respective steroids. Results of these determinations are given in Table II.

REFERENCES

(1) Bićan-Fišter, T., *J. Chromatog.*, **22**, 465(1966).
(2) Umberger, E. J., *Anal. Chem.*, **27**, 768(1955).
(3) Szalikowsky, C. R., O'Brien, M. G., and Mader, W. J., *ibid.*, **27**, 944(1955).

Keyphrases
Steroid mixtures—analysis
Progesterone and estradiol benzoate in oil
Progesterone, testosterone propionate, and estradiol benzoate in oil
Testosterone butyrate and estradiol benzoate aqueous suspension
Progesterone and estradiol benzoate aqueous suspension
TLC separation
Fluorometric analysis
UV analysis

Miotic Activity Produced by Inhibitors of Acetylcholinesterase

By A. Z. BOOTH, J. P. LONG, and K. R. LONG

Various biological activities of bis-phenacyl derivatives were investigated. The picoline derivatives demonstrated potent activity as inhibitors of human red blood cell cholinesterase. The compounds were effective miotic agents in mice and rabbits. Marked differences in toxicity of the positional isomers were noted. The 3-picoline derivative is a very toxic agent in mice, however it is equal to 2-picoline in activity as an inhibitor of acetylcholinesterase. Since the 2-picoline derivative is much less toxic, some mechanism other than inhibition of acetylcholinesterase must be involved to explain the high toxicity of the 3-picoline derivative.

SEVERAL SERIES of bis-quaternary compounds have been prepared that demonstrate potent inhibition of acetylcholinesterase. In general these compounds are much more selective for acetylcholinesterase than they are for pseudo cholinesterase (1). Since the innervation of the circular muscles of the iris is cholinergic and thus the degree of muscle tone is indirectly controlled by functional acetylcholinesterase, the biological activity was evaluated for the potent inhibitors of acetylcholinesterase described in this report. Quaternary compounds that are inhibitors of cholinesterase (*e.g.*, neostigmine) have not been particularly effective as miotic agents, probably because of difficulty of penetration across the cornea. In this series of agents this difficulty apparently was overcome by obtaining compounds that are more active biologically than neostigmine and incorporating into the structure components that would be expected to increase the lipid solubility.

METHODS

The ability to inhibit human red blood cell cholinesterase was measured using a Radiometer titrator type TTT1c titrograph type SBR 2C with Alga syringe buret. The reagents used were as follows: NaCl, 21.6%; NaCl, 22.5%; NaCl, 0.9%; acetylcholine iodide, 0.250 *M*; acetyl-β-methylcholine bromide, 0.5 *M*; Potassium hydrogen phthalate, 0.7351 Gm./200 ml. (2.0 ml. = 36 μmoles NaOH); 0.1 *N* NaOH (carbonate free). Two milliliters of potassium hydrogen phthalate is added to 10.0-ml. distilled water and this solution is titrated under N_2 with 0.1 *N* NaOH. From this titration curve the ordinate of the titrigraph chart is calibrated directly in μmoles NaOH used, which is equivalent to μmoles of liberated acid (cholinesterase activity). The abscissa of the chart gives time in minutes. Values are read as μmoles/ml./min. Red cells are prepared by centrifuging the blood sample at a fixed RFC and then washing twice with normal saline. The washed cells are suspended in an equal volume of normal saline (*i.e.*, 50% solution). To 22.0 ml. of distilled water is added 1.0-ml. whole blood, washed red cells, or plasma. The mixture is allowed to stand for 10 min. at 37–38° to allow for hemolysis and temperature equilibration. A 1.0-ml. amount of 21.6% NaCl is added and the mixture is again allowed to stand for a few minutes so that the temperature will equilibrate. The reaction mixture is then placed on the titrator and N_2 is blown over the top. After adjusting to pH 7.4 (preliminary titration), 1.0 ml. of acetylcholine iodide (0.250 *M*) is added and the reaction rate followed for about 8 min. If the true cholinesterase only is to be measured, 1.0 ml. of acetyl-β-methylcholine (0.5 *M*) is added in place of the acetylcholine iodide. Some samples were found to have too much

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