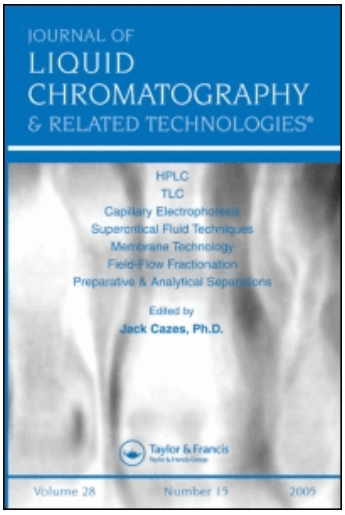

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SIMULTANEOUS ANALYSIS OF ESTRADIOL DIENANTHATE, ESTRADIOL 3-BENZOATE AND TESTOSTERONE ENANTHATE BENZILIC ACID HYDRAZONE IN OILY FORMULATIONS BY GRADIENT-HPLC

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ABSTRACT

A procedure is described for the analysis of estradiol-3-benzoate, testosterone enanthate benzylic acid hydrazone and estradiol dienanthate in oily solution. The multi-step gradient reversed phase ion-pairing HPLC method uses 1-pentane sulfonic acid sodium salt (0.0025 M in 0.1% acetic acid methanolic solution): acetonitrile and water (55 : 30 to 45 : 15 to 0) mobile phase gradient and a 250 x 4.6 mm, 5 micron octadecyl silane column, with 1,2,4,5-tetrachlorobenzene as internal standard. The time required for chromatography is 31 minutes. The method gives relative standard deviations (RSD) of .94% or better for the assay of active ingredients in commercial formulations.

INTRODUCTION

Admixture of testosterone enanthate benzylic acid hydrazone (TEBAH) with estradiol dienanthate and estradiol 3-benzoate in oily

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solution is used as replacement therapy for the control of menopausal symptoms and for estrogen deficiency-induced osteoporosis, where estrogen deficiency is natural or surgically induced.

Extraction of steroid esters from vegetable oil prior to reversed phase HPLC is required to prevent column degradation by absorption of non-polar components. Several procedures were reported in the literature to overcome this problem, including extraction with aqueous alcoholic or acetonitrile solutions of oily formulations either directly (1-3) or following dilution with hexane or similar solvent (5-6), followed by column partition chromatography on either normal (7,8) or silane-treated (9,10) siliceous earth. Absorption of steroid esters on highly efficient silica cartridges, followed by elution with polar solvent was also presented (11).

This paper describes a gradient reversed phase HPLC procedure for the determination of TEBAH, estradiol-3-benzoate and estradiol dienanthate in oily solution following acetonitrile extraction.

EXPERIMENTAL

Apparatus

A fully-automated high-performance liquid chromatography (HPLC) system (Model SP8100, Spectra-Physics, Santa Clara, CA, U.S.A.), operated at ambient temperatures, consisting of a solvent delivery system operating at 2.0 ml/min, an auto-sampler, an air-activated automatic injector equipped with a 10 µl loop, and a variable wavelength UV detector (Model SP8400, Spectra-Physics) set at 215 nm (0.04 a.u.f.s.) was used. The column (250 x 4.6 mm I.D.) was octadecyl silane, chemically bonded to totally porous silica spherical microparticles (5 µ) (Zorbax ODS, Dupont Company, Wilmington, DE, U.S.A.).

Peak retention times and areas were obtained with a reporting integrator (Model SP4100, Spectra-Physics).

Reagents

Estradiol-3-benzoate and testosterone enanthate were U.S.P. reference standards. Estradiol-dienanthate and TEBAH were generously

donated by Merck-Frosst (Pointe-Claire, Quebec, Canada). Tetrachlorobenzene (1,2,4,5) (Aldrich Chemical, Milwaukee, WI) was recrystallysed from methanol. Benzyl benzoate (Sigma Chemical, St.-Louis, MO) and corn oil (Best Foods, Montreal, Canada) were used in the synthetic formulation.

Acetonitrile, methanol (J.T. Baker, Phillipsburg, NJ) and 1-pentane sulfonic acid, sodium salt (Fisher Scientific, Fair Lawn, NJ) were HPLC grade. Water was double-distilled in glass (house system).

Mobile phase gradient

Acetonitrile (A), water (B) and 1-pentane sulfonic acid, sodium salt 0.0025 M in 0.1% acetic acid methanolic solution (C) (filtered through membranes (FH 0.2 µm and FA 0.45 µm, (Millipore, Bedford, MA) and degassed by sparging) were mixed by the solvent delivery system according to the following scheme:

	Time, min.	A%	B%	C%
Isocratic up to	6.00	30.0	15.0	55.0
Linear gradient to	15.00	45.0	0.0	55.0
Hold until	22.5	45.0	0.0	55.0
Then linear gradient to initial composition	23.0	30.0	15.0	55.0
and hold until	31.0	30.0	15.0	55.0

Internal standard solutions

Stock solution:

A solution of 1,2,4,5-tetrachlorobenzene was prepared in acetonitrile at a concentration of 30 µg/ml.

Diluted solution:

An aliquot of internal standard stock solution was diluted in acetonitrile (1 in 10).

Standard preparation:

A standard solution containing estradiol-3-benzoate, TEBAH and estradiol dienanthate was prepared in diluted internal standard solution at the same concentration as in the sample preparation (based on label claim).

Sample preparation:

An aliquot (1.0 ml) of oily formulation was accurately measured and transferred to a 15 ml screw-capped centrifuge tube. Ten (10) ml of acetonitrile was added. The tube was stoppered and shaken for 5 minutes on a horizontal shaker, then centrifuged for 3 minutes at 2000 rpm. The clear acetonitrile layer (top) was transferred as much as possible without disturbing the lower oily layer to a 100 ml volumetric flask. The extraction was repeated 6 more times. All extracts were combined into the 100 ml volumetric flask, after which 10.0 ml of internal standard stock solution was added. The flask was made up to volume with acetonitrile, stoppered and the contents were mixed. Samples were kept in the refrigerator until used.

Recovery study:

Estradiol 3-benzoate - estradiol dienanthate oily solution:

An aliquot of about 10 mg of estradiol-3-benzoate, accurately weighed, was transferred to a 10 ml volumetric flask. Then an aliquot of about 75 mg of estradiol dienanthate, accurately weighed, was transferred to the same flask. A mixture of corn oil, benzyl alcohol and benzyl benzoate (67.5 : 25 : 7.5) was added to the flask to bring it up to volume. The flask was sonicated to dissolve all solid materials. After cooling, the volume was adjusted to the mark (when necessary) with the oily mixture.

Synthetic mixture with TEBAH

An aliquot of about 150 mg of TEBAH, accurately weighed, was transferrd to a 15 ml screw-capped centrifuge tube. One ml of estradiol-

3-benzoate - estradiol dienanthate oily solution was added. The tube was sonicated until all solid materials had dissolved.

This solution was treated as under "Sample Preparation".

Analytical procedure:

Aliquots (10 µl) of standard preparation and sample preparation were successively injected into the chromatograph. Peak areas were measured. The quantities of active ingredients in the sample preparation were calculated using the following formula:

$$C_u = 100 \times C_s \times \frac{R_u}{R_s}$$

where

C_u = Concentration of active ingredient in sample preparation (mg/ml)

C_s = Concentration of active ingredient in standard preparation (mg/ml)

R_u = Area ratio of active ingredient to internal standard in sample preparation

R_s = Area ratio of active ingredient to internal standard in standard preparation

RESULTS AND DISCUSSION

Complete resolution was obtained between estradiol-3-benzoate, TEBAH and estradiol dienanthate as well as from degradation products (testosterone enanthate and other unidentified products) and oil base components. Benzyl alcohol and benzyl-benzoate were eluted much ahead of the first eluted active ingredient (estradiol-3-benzoate), allowing a window for an internal standard (1,2,4,5-tetrachlorobenzene) (Fig. 1, Table 1). A multiple gradient reverse-phase mode was selected to allow separation of the relatively polar compound (estradiol-3-benzoate) from early eluted injection base components (benzyl alcohol, benzyl benzoate),

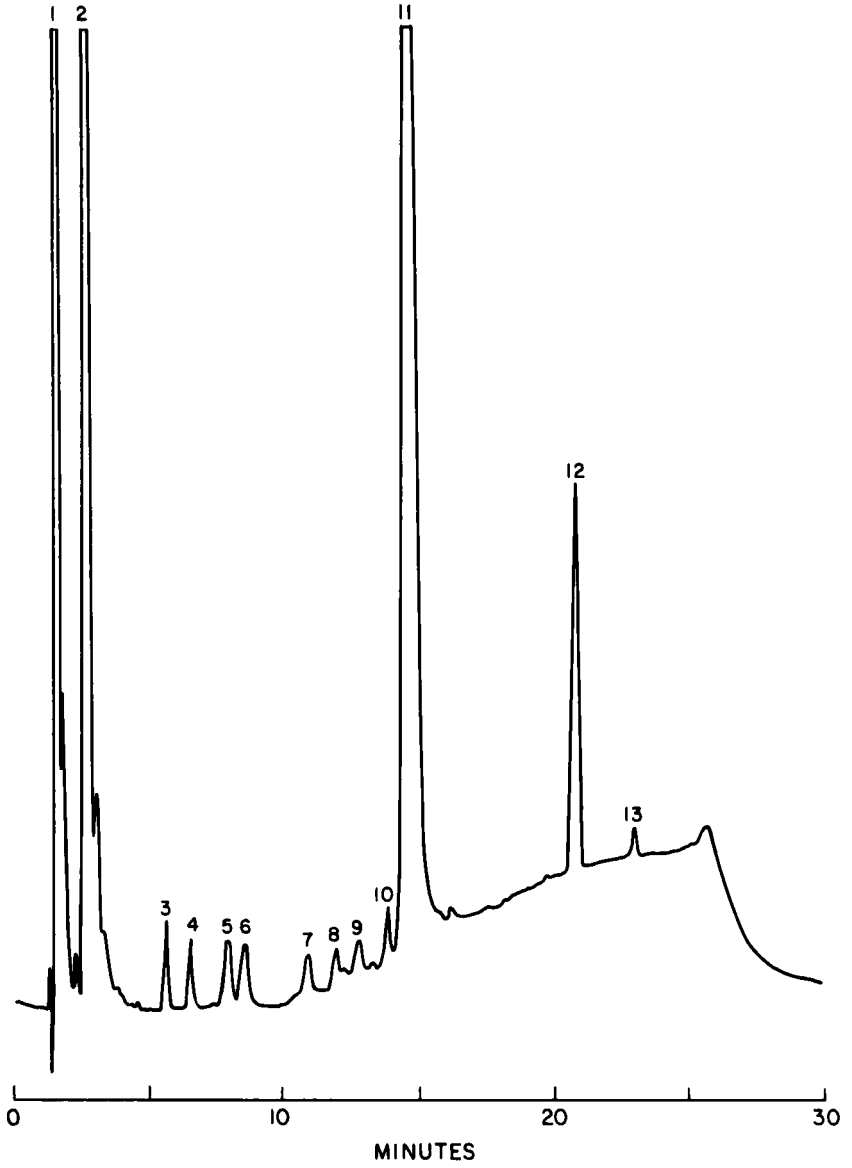

FIGURE 1:

Analysis of a commercial formulation with internal standard. Peaks are numbered according to Table 1.

as well as elution of the highly hydrophobic component (estradiol dienanthate) from oil components within a reasonable time. Addition of ion-pairing agent (1-pentane sulfonic acid, sodium salt) had a significant effect on the peak shape of TEBAH by reducing the tailing to an acceptable value (tailing factor = .873) without affecting elution of any

TABLE 1

Chromatographic Characteristics of Compounds of Interest

No.	Compound	Retention Time	K'	R*	U.V. max‡
	T_o	1.02	-	-	-
1	Benzyl alcohol	1.57	.54	2.9	286
2	Benzyl benzoate	2.74	1.69	7.3	End, 265, 272
3	Tetrachlorobenzene	5.64	4.53	1.5	End, 276, 287
4	Estradiol-3-benzoate	6.56	5.43	1.9	End, 236, 275
5	Unknown No. 1	7.97	6.81	1.0	284
6	Unknown No. 2	8.56	7.39	2.6	284
7	Unknown No. 3	10.97	9.75	2.1	-
8	Unknown No. 4	11.99	10.75	2.1	-
9	Unknown No. 5	12.85	11.60	2.5	-
10	Testosterone enanthate	13.98	12.71	.8	242
11	TEBAH	14.71	13.42	6.0	283
12	Estradiol dienanthate	20.89	19.48	5.0	End
13	Unknown No. 6	23.02	21.57		End

* Resolution factor from following peak $R = 2\left(\frac{t_2 - t_2}{W_1 + W_2}\right)$

‡ Determined using the scanning capability of the UV detector by stopping flow at the apex of the peak.

other compounds. The presence of a small amount of acetic acid (0.1%) was found to be necessary to promote ion-pair formation.

This gradient produced a fairly high and steady baseline drift, even though highly purified HPLC grade reagents were used. However, by proper setting of the automatic integrator parameters, excellent RSDs were found ($\leq$ .86% on area and 1.01% on area ratio) when standard preparation was chromatographed (Table 2).

Linearity of response versus concentration was studied on 6 preparations over the ranges 0.02-0.12 µg for estradiol-3-benzoate, 3-18 µg for TEBAH and 0.15-.90 µg for estradiol dienanthate. Within these ranges, standard curves passed close to the origin and the correlation coefficients were nearly ideal (Table 3).

A silica cartridge extraction procedure (11) did not show any selectivity for separation of estradiol dienanthate from fatty acids, and therefore direct extraction was studied. Acetonitrile was preferred as the extracting solvent because estradiol dienanthate showed a good partition coefficient (53%) under the cited conditions, and also because of its high immiscibility with vegetable oil. It was found that essentially complete recovery was achieved within 6 consecutive extractions. Accuracy and reproducibility were ascertained via the synthetic preparation, and recovery from these preparations was found to be excellent (Table 4).

Quantitative analysis of two lots of a commercial formulation are presented in Table 5. All active ingredient contents are within regulatory limits (90-110%) and RSD is satisfactory ($\leq$ 1.1%). Testosterone enanthate, arising either as a TEBAH synthetic by-product or as a hydrazone hydrolysis product, was found at a level of 2.15% and 3.15% based on TEBAH content, with RSD 2.7% and 1.8% respectively.

Testosterone enanthate was identified by retention time and its UV spectrum, while unknowns No. 1 and 2 could not be identified but were suspected to be related to TEBAH since the UV spectra showed maximum at about 284 nm, characteristic of benzyl derivatives. The unknown No. 6 presumably came from the oil base, since a component of corn oil had the same retention time.

TABLE 2

Reproducibility Study* on Standard Preparations

Compounds	Amount Injected µg	Area	RSD %	Area Ratio	RSD
Internal standard (1,2,4,5-tetrachlorobenzene)	.30	95860	.26	-	-
Estradiol-3-benzoate	.1	55420	.86	.578	1.01
TEBAH	15.0	5872000	.15	61.058	.33
Estradiol dienanthate	.75	228000	.19	2.378	.32

* Average of 5 determinations

TABLE 3

Standard Curves

Compound	No. of Prep.	Range (µg)	Slope	Intercept	Com. Coef.
Estradiol-3-benzoate	6	.02-.12	5.75	0.014	.9996
TEBAH	6	3-18	4.063	.263	.9999
Estradiol dienanthate	6	.15-.90	3.187	.001	1.0000

TABLE 4

Results of Recovery Study

Compound	Added	Found	% Recovery*	RSD
Estradiol-3-benzoate	1.258	1.233	98.0	1.02
TEBAH	149.40 153.57 147.03 150.41	148.5 154.1 145.6 150.1	99.7	.57
Estradiol dienanthate	7.687	7.558	99.4	.78

* Average of 4 determinations

TABLE 5

Results of Analysis of Commercial Formulations

Compounds	A		B	
	% Label	RSD %	% Label	RSD %
Estradiol-3-benzoate	94.1	1.07	95.5	.48
Testosterone enanthate*	2.15	2.7	3.13	1.8
TEBAH	94.6	.88	95.4	.94
Estradiol dienanthate	100.4	.77	99.9	.64

Average of 4 determinations

* Relative to TEBAH label claimed

CONCLUSION

This multi-step, gradient reversed phase HPLC method for the analysis of estradiol-3-benzoate, testosterone enanthate benzylic acid hydrazone and estradiol dienanthate in oily solution is fast, specific and accurate.

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