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Note

Determination and stability study of oestradiol benzoate in a pharmaceutical ointment by high-performance liquid chromatography

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Oestradiol benzoate (OB) is a well-known oestrogen used in hormonal therapy[1]. Our study concerned the assay of a pharmaceutical ointment that contains OB and an antibacterial agent (tyrothricine) as active substances. This preparation is used to prevent and cure nipple-chapping, which may appear during breast-feeding.

Gas chromatography (GC)[2] and high-performance liquid chromatography (HPLC)[3–7] techniques have already been described for the determination of oestrogenic substances in pharmaceutical dosage forms. The previously reported HPLC methods involve both normal-phase (NP)[4] and reversed-phase (RP)[3–7] systems with spectrophotometric[3–6] or electrochemical[7] detection. The latter is more sensitive but is only suitable for the detection of steroids with a free phenolic group. Among all these procedures, no particular care has been provided for the determination of degradation products released by the drugs, especially in the case of steroid esters.

This report deals with different chromatographic methods elaborated in order to determinate OB and its degradation products (oestradiol and benzoic acid) resulting from ester bond cleavage and appearing during storage.

EXPERIMENTAL

Reagents and chemicals

All chemicals and solvents were of analytical reagent grade. β-Oestradiol-3-benzoate (OB) was obtained from Diosynth (St. Denis, France) and obeyed the European Pharmacopoeia requirements. β-Oestradiol, benzoic acid and cyclohexane carboxylic acid were purchased from Aldrich (Strasbourg, France).

Components of the studied ointment were OB (60 μg per gram) and tyrothricine as active substances, and starch, ethanol, glycerol, water and poly(oxyethylene glycol)s as formulating aids.

Standard and sample preparations

Freshly made solutions of OB (60 mg/100 ml), oestradiol (10 mg/100 ml) and benzoic acid (10 mg/100 ml) in ethanol were used as stock solutions and appropriately diluted with the same solvent.

Oestradiol benzoate and free oestradiol were determined as follows: an equal

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weight of ointment (5 g) was introduced into each of four 50-ml vials. These samples were spiked with 0.00, 0.25, 0.50 and 0.75 ml, respectively, of the OB stock solution, and 10 ml of dichloromethane–ethanol (20:80, v/v) were added to each vial. After vigorous shaking [maximum speed for 15 min on a mechanical shaker (Model SA-31; Yamato Tokyo, Japan)], the vials were centrifuged at 5000 rpm for 10 min, and the organic phase was transferred into 25-ml flasks. The extraction was repeated once and the volume made up to 25 ml with the same solvent mixture. An aliquot of this solution was injected into the chromatograph for the determination of both OB and oestradiol.

Benzoic acid was determined in the following way: to a 5-g sample of ointment were added 50 μl of 1 mg/ml cyclohexane carboxylic acid solution in ethanol, 5 ml of 0.1 *M* hydrochloric acid and 15 ml of diethyl ether. The mixture was mechanically shaken, under the same conditions as above; the extractive step was repeated twice and the organic layers were collected, dried over sodium sulphate, and evaporated to dryness. The dried residue was redissolved in 1 ml of acetone and 0.5 ml of 1.5 mg/ml 4-bromomethyl-7-methoxycoumarin (BrMmc) solution in acetone, 0.5 ml of 1 mg/ml 18-crown-6-ether solution in acetone and 20 mg of potassium carbonate were added[8]. The reaction mixture was heated at 70°C for 30 min, then cooled in ice. An aliquot was injected into the chromatograph.

Chromatographic conditions

The HPLC system consisted of a ternary solvent-delivery pump (Model SP 8700; Spectra-Physics, Santa Clara, CA, U.S.A.), an injection valve with a 20-μl sample loop (Model 7125; Rheodyne, Cotati, CA, U.S.A.) and a UV–VIS detector (Model LC 871, Pye Unicam, Cambridge, U.K.). For amperometric detection, a thin-layer electrolytic cell (Model LCC 231; Merck-Clevenot, Nogent-sur-Marne, France), fitted with glassy-carbon working and auxiliary electrodes and a reference saturated calomel electrode (SCE), was used in connection with an electronic control unit (Model E 230; Merck-Clevenot).

The reversed-phase column was prepacked with LiChrosorb RP-18 (Hibar E.C. 250 × 4 mm I.D., 7 μm; Merck-Clevenot). A pre-column (30 × 4 mm I.D.) packed with LiChrosorb RP-18 (40 μm) was used in-line for all chromatographic analyses.

Mobile phases were filtered through a 0.6-μm microfilter (Type HVLP; Millipore, Bedford, MA, U.S.A.) and deaerated before use.

The different mobile phase compositions and detection conditions are given in Table I.

Chromatograms were recorded and peak areas were calculated with an integrator (Model ICR-1; Intersmat, Courtry, France).

Calculations

The amount of OB in ointment samples was calculated by the method of continuous variation of standard addition at constant total volume. The equation of the straight line obtained by plotting peak areas (y axis) *versus* micrograms of OB added to the samples (x axis) permits the calculation of the intercept of the curve with the x axis. The unknown OB amount is deduced from this latter value. The amount of β-oestradiol was determined by comparison with an external standard.

TABLE I

CHROMATOGRAPHIC SYSTEMS USED FOR THE DETERMINATION OF THE DIFFERENT SOLUTES

Solute	*Mobile phase*	*Flow-rate (ml min^{-1})*	*Detection*	*Capacity factor*
Oestradiol benzoate	Methanol–water (90:10, v/v)	1.5	Spectrophotometry λ = 230 nm	4.6
Oestradiol	Methanol–water–acetic acid (60:35:5; v/v/v) + lithium perchlorate (1 g l^{-1})	1.2	Amperometry E = +0.85 V *vs.* S.C.E.	2.1
Benzoic acid	Methanol–water (80:20; v/v)	1.0	Pre-column derivatization and spectrophotometry λ = 328 nm	2.7
Cyclohexane carboxylic acid				3.9

Benzoic acid was quantified with a standard solution under the same conditions and the internal standard method for calculation.

Simultaneous determination of these three compounds in the ointment was successfully carried out after 0, 1, 2, 3 and 4 year storage periods.

RESULTS AND DISCUSSION

Some problems arose during the development of the analytical procedure for the determination of OB in the studied pharmaceutical ointment. A loss of OB was observed even in a freshly manufactured sample. Furthermore, this incomplete recovery increased with storage. Similar facts have been reported in the literature: the recovery of OB and other corticosteroids from a variety of cosmetic samples was in the range from 92% to nearly 100%[4,6]. The formation of bonds between the hydrophobic steroid and the hydrocarbon-like excipients has been claimed to explain the incomplete yield occurring during extraction of oestrogenic drugs from creams[9].

In our study, no free oestradiol was detected in a fresh ointment. So, we searched for an extractive procedure that would improve the OB recovery. Best results were obtained by using a mixture of organic solvents (dichoromethane and ethanol) and by repeating the extractive step twice. However, the OB amount calculated by comparison with an external standard reached no more than 95% of the theoretical value (56.6 μg/g; n = 5; C.V. = 1.7%). A third extractive step had no significant influence on the yield.

As we failed to increase this value, we developed an appropriate method of standard addition[10]. Equal weights of the ointment were spiked with known and increasing amounts of OB. Each sample was extracted by the previously elaborated procedure and made up to the same volume. The resulting experimental value ap-

peared close to the theoretical amount (60.3 μg/g; n = 5, C.V. = 1.85%). This method suppresses any analytical error resulting from interference by OB and other ointment component. Furthermore, the procedure was time-saving compared with more elaborate clean-up methods that include one or more steps usually with the intention of separating the formulating aids from the active component by liquid–solid extraction on a silica gel column[6].

We used a chromatographic system for OB determination which shows good selectivity towards this substance. Other ointment components lack U.V. absorbance at 230 nm or are polar, so they are eluted almost immediately after the void volume. However, the reversed-phase column was flushed with pure methanol before elution with another mobile phase.

We have elaborated two other specific chromatographic systems for the iden-

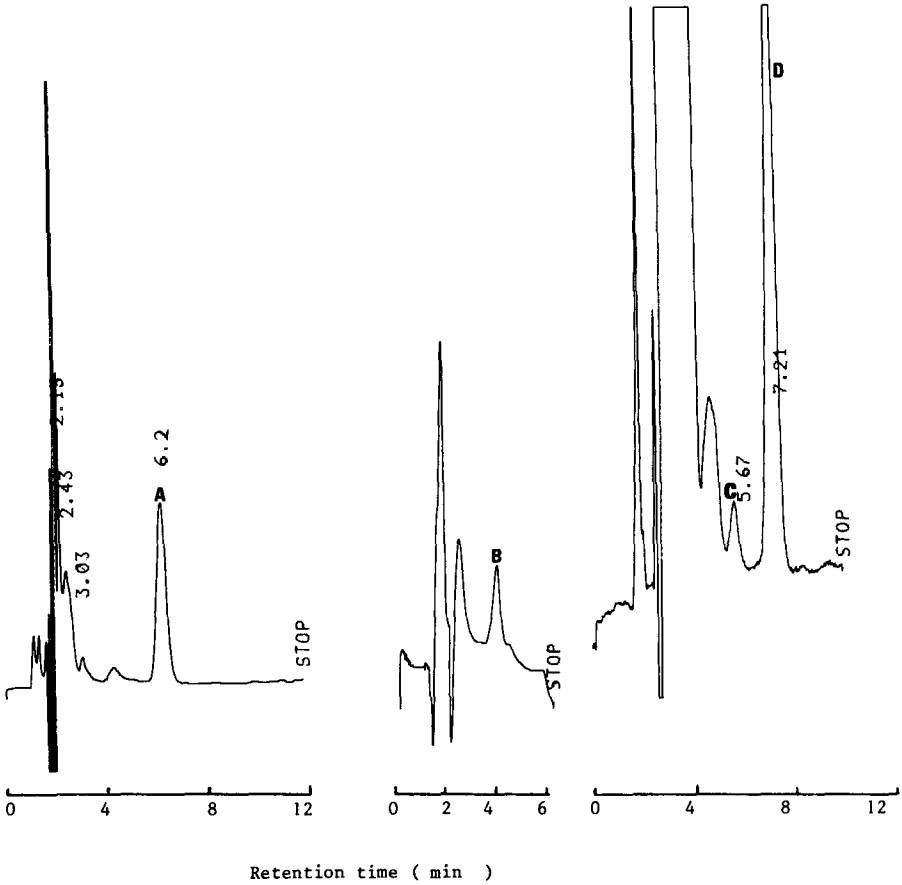

Fig. 1. Chromatograms of oestradiol benzoate (A), oestradiol (B) and derivatized benzoic acid (C) [with cyclohexane carboxylic acid (D) as internal standard] in an ointment extract. These compounds are eluted on a LiChrosorb RP-18 (7 μm) column (250 × 4 mm I.D.) with methanol–water (90:10, v/v) for A, methanol–water–acetic acid (60:35:5, v/v/v) for B, and methanol–water (80:20, v/v) for C and D. Detection was either by UV spectrophotometry (A, C and D) or by amperometry (B). For more details, see Table I. Injected amounts in the HPLC system were, respectively, 200 ng of A, 20 ng of B, 2 ng of C and 40 ng of D.

tification and quantification of the presumed degradation products released by the ester bond cleavage of OB (*i.e.* oestradiol and benzoic acid). Detection modes in these systems were chosen for their sensitivity: electrochemical detection, which relies on the free phenolic group of oestradiol, and pre-column labelling of the carboxylic group of benzoic acid with bromomethylmethoxycoumarin, as previously described for other compounds of biological interest[8]. Their low detection levels permit the quantification of degradation products and oestradiol benzoate with the same sample size. Typical chromatogram are shown in Fig. 1.

The amounts of free oestradiol so found after different storage periods correlated well with the remaining OB amounts (see Fig. 2). But the benzoic acid concentrations measured were not those expected from the loss of OB during storage: they reached no more than 0.5 μg per gram of ointment instead of a theoretical value of 7.2 $\mu g\ g^{-1}$ after 4 years. This may result from the formation of bonds between the free carboxylic group and polar groups of other components. Further investigations are proceeding in order to explain this phenomenon.

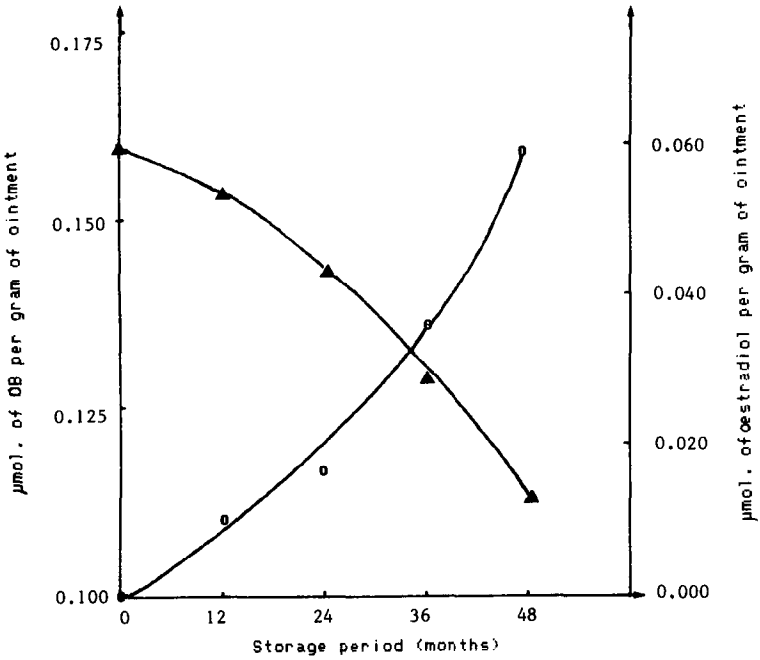

Fig. 2. Amounts of oestradiol benzoate (▲) and free oestradiol (○) found after different storage periods.

Storage period (months)	*Amount of (µmol per gram of ointment)*		
	Oestradiol benzoate (MW = 376.5)	*Oestradiol (MW = 272.4)*	*Sum*
0	0.159	0.000	0.159
12	0.154	0.010	0.164
24	0.143	0.016	0.159
36	0.128	0.036	0.164
48	0.113	0.059	0.172

The methodology developed is well suited to administrative requirements concerning stability studies of a drug contained in a pharmaceutical dosage form.

CONCLUSION

A fast and reliable HPLC technique has been elaborated for the determination of OB in a pharmaceutical ointment. The quantitation by the method of continuous variation of standard addition to the samples avoids a time-consuming extractive procedure and suppresses any interference between OB and the initial matrix. Furthermore, the degradation products appearing from OB (oestradiol and benzoic acid) were identified with use of specific chromatographic systems, and the measured amounts of free oestradiol were correlated with the loss of OB during a 4-year storage period.

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