Table I—Reproducibility of Recovery of Amitriptyline and Metabolites from Spiked Hemolyzed Whole Blood

Compounds Added to Whole Blood	Normalized Peak Heights × 10^2[a]					
	25 ng[b]	100 ng[b]	200 ng[b]	0.5 ml[c]	1.5 ml[c]	2.0 ml[c]
I	1.69 ± 0.05	1.83 ± 0.06	1.73 ± 0.04	1.78 ± 0.06	1.57 ± 0.06	1.37 ± 0.08
II	1.53 ± 0.06	1.57 ± 0.06	1.53 ± 0.03	1.56 ± 0.05	1.47 ± 0.03	1.38 ± 0.06
IV	0.56 ± 0.06	0.61 ± 0.02	0.64 ± 0.01	0.65 ± 0.01	0.55 ± 0.03	0.48 ± 0.02
V	0.18 ± 0.02	0.15 ± 0.01	0.16 ± 0.004	0.18 ± 0.005	0.14 ± 0.004	0.13 ± 0.007
VI	0.21 ± 0.02	0.20 ± 0.01	0.23 ± 0.03	0.23 ± 0.005	0.19 ± 0.01	0.18 ± 0.01

[a] Mean ± *SD*, $n = 5$ for columns 1–3, $n = 4$ for columns 4–6. [b] Amount of I and metabolites added to 1.0 ml of blank, hemolyzed whole blood. [c] Volumes of blank, hemolyzed whole blood to which 100 ng of each component was added.

several months for quantitation of I and all of its metabolites. The use of a water-jacket to maintain column eqilibrium should not be necessary if laboratory ambient temperature fluctuates 3° or less daily.

Plasma samples can be analyzed satisfactorily using the same procedure as that followed for blood samples. As evidenced by the reproducibility of the normalized peak height values of I and the fact that there was no trend in these values with concentration, adsorption to glassware, if it occurred, did not present a problem with the calibration of the assay. This is in contrast to other extraction procedures where adsorption to glassware has resulted in erratic recoveries (7). Butanol was added to the extracting solvent to prevent emulsification and to improve extraction of the more polar metabolites and the internal standard. It is likely that this short-chain alcohol acts like isoamyl alcohol in decreasing adsorption of basic drugs to glass. When standard solutions were assayed, all methanol was evaporated off before the addition of whole blood, as it otherwise affected the cleanliness of the extract. Maximum recovery depended on careful attention to every step of the extraction, particularly the pooling of the organic phase, evaporation, and final vortexing.

Although the addition of bases to mobile phases used with silica columns is known to decrease column life (8), the low concentration of aqueous ammonium hydroxide used in this procedure caused little deterioration of the packing material. A greater problem arose from column contamination by sample extracts, resulting in broadening or even splitting of the peaks. This condition was rectified periodically by removing the top few millimeters of packing material and replacing it with glass microbeads. The use of a guard column packed with pellicular silica should circumvent this problem.

The present method offers several advantages over some previously published procedures. The extraction is simple and rapid, the chromatography is short (15 min), yet allows good separation of I and its known active metabolites. The sensitivity is good, which should allow pharmacokinetic studies to be carried out; the procedure can be applied to either whole blood or plasma. For high-clearance compounds such as I, whole blood measurements may be preferable, obviating the need for determining the red cell to plasma partition coefficient, which may be concentration-dependent.

REFERENCES

(1) J. W. Hubbard, K. K. Midha, J. K. Cooper, and C. Charette, *J. Pharm. Sci.*, **67**, 1571 (1978).

(2) H. E. Hamilton, J. E. Wallace, and K. Blum, *Anal. Chem.*, **47**, 1139 (1975).

(3) L. A. Gifford, P. Turner, and C. M. B. Pare, *J. Chromatogr.*, **105**, 107 (1975).

(4) J. Vasiliades and K. C. Bush, *Anal. Chem.*, **48**, 1708 (1976).

(5) W. A. Garland, R. R. Muccino, B. H. Min, J. Cupano, and W. E. Fann, *Clin. Pharmacol. Ther.*, **25**, 844 (1979).

(6) F. L. Vandemark, R. F. Adams, and G. J. Schmidt, *Clin. Chem.*, **24**, 87 (1978).

(7) H. F. Proless, H. J. Lohmann, and D. G. Miles, *ibid.*, **24**, 1948 (1978).

(8) A. Wehrli, J. C. Hildenbrand, H. P. Keller, R. Stampfli, and R. W. Frei, *J. Chromatogr.*, **149**, 199 (1978).

ACKNOWLEDGMENTS

Supported by a grant from the National Institute of Aging AG01340. Dr. Blaschke is a Burroughs Wellcome Scholar in Clinical Pharmacology, and recipient of a Research Career Development Award (GM00407) from the National Institutes of Health. Dr. Schulz was supported by the Fonds National Suisse de la Recherche, the Holderbank Foundation, and the Merck Foundation. Dr. Giacomini is supported by an NRSA (GM07344) from the National Institutes of Health.

The authors are grateful to Anthony Abang for technical assistance, and to Linda Halloran for preparation of the manuscript.

NOTES

Analysis of Conjugated Estrogens in a Vaginal Cream Formulation by Capillary Gas Chromatography

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Received June 9, 1981, from the *Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, V6T 1W5, Canada.* Accepted for publication August 26, 1981.

Abstract □ A capillary gas-chromatographic method is described for the quantitative analysis of nine equine estrogens in a vaginal cream formulation. The sodium sulfate ethers of the estrogens were selectively extracted from the formulation, subjected to enzyme hydrolysis, and derivatized to their oxime-trimethylsilyl esters. Resolution of the resulting derivatives was achieved on a short (15 m) capillary column, wall-coated with cyanopropylmethyl silicon stationary phase.

Keyphrases □ Estrogens—conjugated, analysis of a vaginal cream formulation by capillary gas chromatography □ GC—capillary, analysis of conjugated estrogens in a vaginal cream formulation □ Vaginal cream formulation—conjugated estrogens, analysis by capillary gas chromatography

The pharmaceutical product known as conjugated estrogens, is a mixture of nine or more equine estrogens isolated from the urine of pregnant mares (1). As their sodium sulfate salts, they have been used in tablet, injectable, and cream formulations for relieving symptoms associated with menopause. Application of the vaginal cream

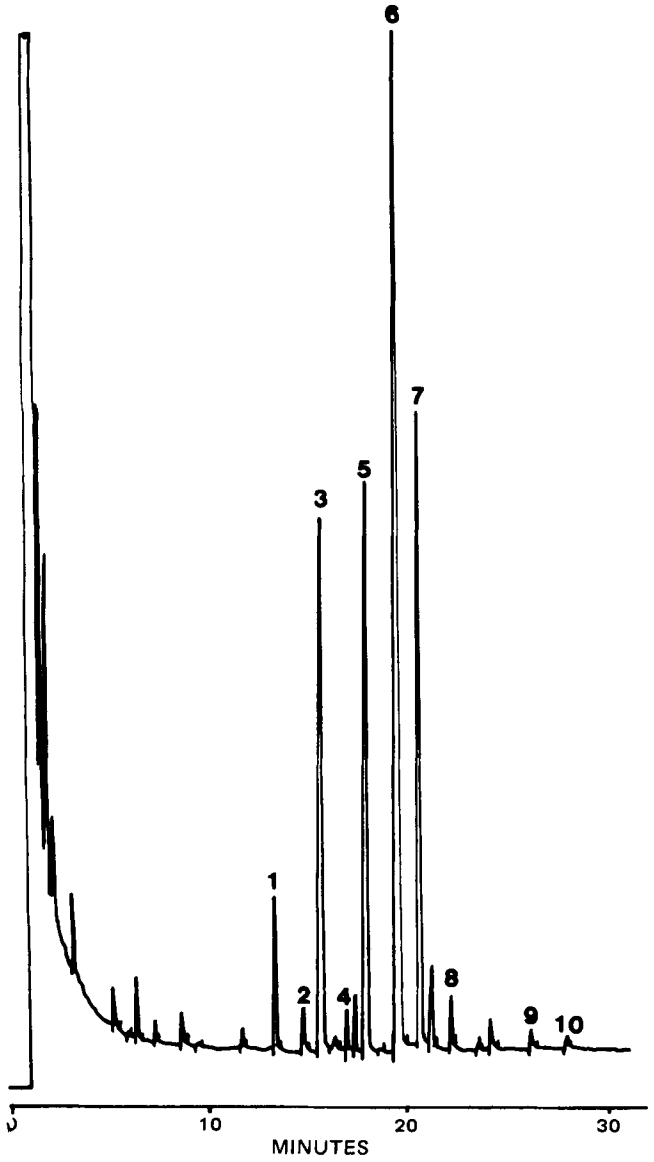

Figure 1—*Chromatogram of equine estrogens as their oxime–trimethylsilyl derivatives derived from a vaginal cream formulation. Key: 1, 17α-estradiol; 2, 17β-estradiol; 3, 17α-dihydroequilin; 4, 17β-dihydroequilin; 5, ethinyl estradiol (internal standard); 6, estrone; 7, equilin; 8, 17α-dihydroequilenin; 9, 17β-dihydroequilenin; and 10, equilenin.*

has been shown to produce a marked improvement in vaginal and vasomotor symptoms (2), and it has been noted that estradiol and estrone plasma levels significantly increased after a single dose. Since estrogens are readily absorbed into the bloodstream after local application, an accurate and selective method for quality assessment of the cream is as essential as one for the tablet and injectable formulations.

At the present time, the assay contained in the United States Pharmacopeia (3) is a colorimetric procedure that quantifies only the two major steroids, estrone and equilin. A gas chromatographic (GC) procedure is specified only for identification of the steroid mixture. Monographs in USP relate to the tablet formulation only. More selective packed column (4, 5) and capillary column (6) GC methods have been reported for the quantitative analysis of the individual steroids in conjugated estrogens. However, these methods have been suitable only for tablet and injectable formulations.

Table I—Analysis of Vaginal Cream Formulation [a]

Component	Potency[a]	Percentage
17α-Estradiol	0.0196	3.14
17β-Estradiol	0.0054	0.86
17α-Dihydroequilin	0.0941	15.07
17β-Dihydroequilin	0.0074	1.18
Estrone	0.3122	49.99
Equilin	0.1497	23.97
17α-Dihydroequilenin	0.0113	1.81
17β-Dihydroequilenin	0.0015	0.24
Equilenin	0.0233	3.73
Total	0.6245	99.92

[a] Expressed as milligrams of the sodium sulfate salts in each gram of cream (label claim: 0.625 mg/g). Average of two assays.

The quantitative method described herein is a modification of a capillary GC assay described previously (6) for tablet and injectable formulations.

EXPERIMENTAL

A capillary gas chromatograph[1] operated in the split mode was used with a flame ionization detector and a data terminal[2] for all measurements. A commercial 15 m × 0.25-mm borosilicate column[3], wall-coated with cyanopropylmethyl silicon liquid phase (Silar 10C), was used with a helium carrier gas flow of 0.8 ml/min through the column and a split vent flow of 40 ml/min. The column inlet pressure was 10 psi. The injector and detector temperatures were 250° and the oven was programmed from 170° for 7 min to 220° at 2.3°/min and was maintained at the upper temperature until elution of the last peak (equilenin).

Materials and Supplies—Methylene chloride, chloroform, acetic acid, sodium acetate, and sodium chloride were reagent grade quality. *N,O*-Bis(trimethylsilyl)trifluoroacetamide and pyridine were silylation grade[4]. Sulfatase enzyme[5], ethinyl estradiol[6], hydroxylamine hydrochloride[7], and equine estrogens[8] were used without further purification.

Sample Preparation—An accurately weighed sample of the vaginal cream, equivalent to ~1 mg of conjugated estrogens, was transferred directly into a 50-ml screw-capped centrifuge tube. Ten milliliters of a 10% solution of sodium chloride in water were added and the tube was shaken thoroughly. The suspended cream was washed twice with 25-ml portions of methylene chloride and the organic phase was discarded after centrifugation.

Nitrogen was bubbled through the aqueous phase to remove any residual methylene chloride until its odor was no longer apparent. To the aqueous extract was added 15 ml of acetate buffer (pH 5.2, 0.02 *M*), 2000 U of sulfatase enzyme, and the mixture was incubated for 30 min at 45°. Following hydrolysis of the sodium sulfate esters, a 0.2-ml aliquot of the internal standard, ethinyl estradiol (1.0 mg/ml), in chloroform was added and the mixture was shaken for 15 min on a rotary tube tumbler[9]. After centrifuging, the chloroform layer was separated and evaporated under a gentle nitrogen stream. The equine estrogens were then converted to their oxime–trimethylsilyl derivatives by the addition of 200 μl of hydroxylamine hydrochloride in pyridine (2.0%) and heating the solution in a screw-capped conical vial[10] for 30 min at 70°. After conversion of the keto-steroids to their oxime derivatives, the remaining hydroxy functions were silylated by the addition of 150 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide and further heating the vials for 10 min at 70°. A 2-μl aliquot was used for analysis.

RESULTS AND DISCUSSION

The chromatogram shown in Fig. 1 is representative of the steroid profile obtained from the analysis of a vaginal cream formulation. The identity of each steroid was established previously (6) by comparison with authentic equine estrogens. The resolution of the capillary column is sufficient to separate all the estrogen steroids along with the internal standard in ~28 min.

[1] Model 5830A, Hewlett-Packard, Avondale, Pa.
[2] Model 18850A, Hewlett-Packard, Avondale, Pa.
[3] Alltech Associates, Rockford, Ill.
[4] Pierce Chemical Co., Rockford, Ill.
[5] Type H-2, Sigma Chemical Co., St. Louis, Mo.
[6] Sigma Chemical Co., St. Louis, Mo.
[7] British Drug Houses, London, England.
[8] Ayerst Pharmaceuticals, Montreal, Quebec, Canada.
[9] RotoRack, Fisher Scientific Co. Ltd., Vancouver, B.C., Canada.
[10] Reacti-Vial, Pierce Chemical Co., Rockford, Ill.

The quantitation of each of the equine estrogens separated in this fashion was accomplished by using the relative response ratios of each estrogen as compared to the internal standard, ethinyl estradiol. For such determinations, the response ratio and linearity of response were determined by injection of six aliquots of each individual steroid at levels bracketing the anticipated quantity in the mixture with a constant amount of internal standard. For all such measurements, the correlation between peak area ratios and concentrations was 0.998 or better.

Extraction of the water soluble sodium sulfate salts of the equine estrogens required the addition of sodium chloride to suppress emulsion formation. The methylene chloride extraction effectively removed lipid soluble formulation excipients as evidenced by a chromatogram that was essentially devoid of extraneous peaks other than those of the equine estrogens. Subsequent hydrolysis of the sulfate conjugates with 2000 U of sulfatase enzyme had earlier been reported (5) as sufficient for hydrolysis of 1 mg of the conjugates, even in the presence of small amounts of phosphates.

The quantitative assay data obtained from the analysis of two aliquots of a vaginal cream formulation is given in Table I. The quantities of estrone and equilin are within the limits specified for conjugated estrogens in the United States Pharmacopeia (3). The total potency determined indicates that the product was 99.9% of labeled claim.

In summary, this method represents the first quantitative procedure for the total analysis of the steroid composition in an estrogen vaginal cream formulation.

REFERENCES

(1) R. Roman, C. A. Yates, J. F. Miller, and W. J. A. VandenHeuvel, *Can. J. Pharm. Sci.*, **10**, 8 (1974).

(2) R. N. Dickerson, R. Bressler, C. D. Christian, and H. W. Hermann, *Clin. Pharmacol. Ther.*, **26**, 502 (1979).

(3) "The United States Pharmacopeia," 20th rev., Mack Publishing, Easton, Pa., 1980.

(4) R. Johnson, R. Masserano, R. Haring, B. Kho, and G. Schilling, *J. Pharm. Sci.*, **64**, 1007 (1975).

(5) K. M. McErlane and N. M. Curran, *ibid.*, **66**, 523 (1977).

(6) G. K. Pillai and K. M. McErlane, *ibid.*, **70**, 1072 (1981).

ACKNOWLEDGMENTS

This research was supported by a grant from the Natural, Applied, and Health Sciences, University of British Columbia.

The authors would like to thank Ayerst Pharmaceuticals, Montreal, Canada for the generous gift of equine estrogens and formulations required.

Effect of Some Formulation Adjuncts on the Stability of Benzoyl Peroxide

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Received June 8, 1981, from the *University of Houston, College of Pharmacy, Department of Pharmaceutics, Houston, TX 77030.* Accepted for publication August 26, 1981.

Abstract □ The stability of benzoyl peroxide in polyethylene glycol ointment base and some liquid vehicles (acetone, ethanol, propylene glycol, and their mixtures) was studied. Some solutions also contained an additional ingredient (acetanilide, benzoic acid, chlorhydroxyquinoline, and hydroxyquinoline) as a possible stabilizer. Benzoyl peroxide decomposed very fast (first-order K value 0.028 day^{-1} at 24°) in polyethylene glycol ointment base. At 50°, the potency of benzoyl peroxide in polyethylene glycol ointment base decreased to <1% in 5 days. Decomposition in solutions is complex. Considering acetone as a standard vehicle, ethanol improved the stability of benzoyl peroxide and propylene glycol had an adverse effect on the stability. Of the stabilizers studied, only chlorhydroxyquinoline improved the stability.

Keyphrases □ Benzoyl peroxide—stability, effect of some formulation adjuncts □ Formulations—benzoyl peroxide, effect of some formulation adjuncts on stability □ Keratolytics—benzoyl peroxide, effects of some formulation adjuncts on stability

Benzoyl peroxide formulations are used extensively for the treatment of acne; however the literature on the stability of benzoyl peroxide is scarce. Gruber *et al.* (1) studied the stability of some commercial lotions containing I but the formulations' excipients were not reported. According to that report, benzoyl peroxide usually decomposes to benzoic acid and carbon dioxide.

The stability of benzoyl peroxide in pharmaceutical gels was reported by Bollinger *et al.* (2). These authors recommended the use of sodium hydroxide over triethanolamine as a neutralizing agent. Moreover, gels containing some acetone were reported to be very stable (even at higher temperature) *versus* ethanol which had an adverse effect on the stability of benzoyl peroxide. These studies used a selective titrimetric analysis method (3).

A stability-indicating assay method using high-pressure liquid chromatography (HPLC) was reported recently (4). The report questioned the accuracy of the titrimetric method (3) which is also official in the USP (5). The latest USP–NF edition (6) recommends the same titrimetric method of analysis for both hydrous benzoyl peroxide and its lotion.

The purpose of these investigations was to study the effect of some formulation adjuncts, polyethylene glycol ointment USP (7), some liquid vehicles, and some possible stabilizers, on the stability of benzoyl peroxide.

EXPERIMENTAL

Chemicals and Reagents—All the chemicals and reagents were either USP, NF, or ACS grade and were used without further purification. Benzoyl peroxide granules[1] and hydrous benzoyl peroxide granules[2] were used as received.

Apparatus—The HPLC[3] was equipped with a multiple wavelength detector[4], a recorder[5], and a digital integrator[6].

Column—A nonpolar column[7] (30 cm × 4-cm i.d.) was used.

Chromatographic Conditions—The mobile phase contained 60% (v/v) of acetonitrile and 0.8% (v/v) of glacial acetic acid in water. The flow rate was 2.5 ml/min and the temperature was ambient. The sensitivity was set at 0.04 (254 nm) and the chart speed was 30.5 cm/hr.

Preparation of Ointments for Stability Studies—A 2.0% (w/w)

[1] Aldrich Chemical Co., Milwaukee, Wis.

[2] Pennwalt Corp., Buffalo, N.Y.; generously supplied by Alcon Laboratories, Fort Worth, Texas.

[3] Model ALC 202 equipped with a U6K universal injector, Waters Associates, Milford, Mass.

[4] Spectroflow monitor SF770, Schoeffel Instrument Corp., Westwood, N.J.

[5] Omniscribe 5213-12, Houston Instruments, Austin, Texas.

[6] Autolab minigrator, Spectra-Physics, Santa Clara, Calif.

[7] μBondapak C_{18} (catalog no. 27324), Waters Assoc., Milford, Mass.

0022-3549/82/0500-0585$01.00/0