rate of all types of aminocephalosporins was not significantly affected by a salt concentration.

From the present results, it is suggested that the dissolution of the aminocephalosporins studied cannot be a rate-limiting step in their absorption when they are ingested with sufficient water, as concluded previously (1) for aminopenicillins. The reduced bioavailability reported (12) for the least soluble cephaloglycin may be the result of the instability of the antibiotic at gastric and intestinal pH (3, 12) and of relatively slow GI membrane permeability of the dissolved cephaloglycin itself (13).

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# Separation and Quantitation of Esterified Estrogens in Bulk Mixtures and Combination Drug Preparations Using High-Performance Liquid Chromatography


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Received March 15, 1978, from the *Quality Control Department, Hoffmann-La Roche, Inc., Nutley, NJ 07110.* Accepted for publication August 21, 1978.



**Abstract** □ A high-performance liquid chromatographic method for esterified estrogens is described. By using a facile acid hydrolysis extraction procedure for the sample preparation, the compounds are chromatographed as their free phenolic forms. The separation of structurally similar compounds, such as equilenin, equilin, estrone, and estradiol, was achieved with a reversed-phase column and a methanol–water mobile phase. Several samples of bulk mixtures and tablets were assayed; the results compared favorably with those obtained using the USP XIX method. The method was rapid, and the detector response was linear over a wide concentration range. A relative standard deviation of ±5% indicates the reliability and accuracy of the proposed method.



**Keyphrases** □ Estrogens, esterified—high-performance liquid chromatographic analysis in prepared solutions □ High-performance liquid chromatography—analysis, esterified estrogens in prepared solutions □ Hormones—esterified estrogens, high-performance liquid chromatographic analysis in prepared solutions


A combination drug preparation containing chlordiazepoxide base and esterified estrogens[1] is assayed for estrone and equilin by the USP colorimetric procedure. This modified Kober reaction procedure is quite lengthy (1) and complicated. Quantitation is concerned mainly with the proper ratio of the sulfate esters of estrone and equilin, calculated on the basis of the total estrogen content.

## BACKGROUND

The present compendial analytical technique is remiss in that minor constituents of these complex estrogenic substances, such as equilenin, dihydroequilenin, dihydroequilin, and estradiol, are not differentiated readily (2–4). In an attempt to replace the cumbersome USP assay, many steroid separations have been reported.

In TLC, emphasis is placed on the use of silver nitrate-impregnated silica gel plates and a variety of solvent systems for resolution of the equine estrogens and their sulfates (5, 6).

Reversed-phase partition liquid chromatographic separation of estrone and equilin was achieved (7) by using an argentated mobile phase. The presence of small amounts of silver ion produced well-resolved equilin and estrone peaks (7).

Reported GLC determinations of urinary estrogens require preliminary purification and isolation on adsorption columns (8) and/or thin-layer plates (9, 10). These isolated free estrogens are derivatized subsequently to a more stable form amenable to the high temperatures encountered in GLC. Since these compounds are heat-labile substances, conversion to their silyl ethers is mandatory (11–13). It is this particular characteristic that makes open-column liquid chromatographic (14–16) and high-performance liquid chromatographic (HPLC) applications more practical and less time consuming (17–25).

Published HPLC determinations of conjugated and esterified estrogens in commercial dosage formulations are few (20, 22, 24). Most described methods are not applicable to this product because derivatizations with heat are used prior to the final chromatographic analysis (22–25).

These problems motivated the development of the assay described in this report. An analytical separation of the hydrolyzed esterified estrogens in various dosages was obtained by HPLC *via* a reversed-phase column and a methanol–water mobile phase.

Data obtained from statistical evaluations show that reliability and precision are attained using the method; the total estrogen content is easily quantitated by adding the individual estrogens.

## EXPERIMENTAL

**Apparatus**—A constant pressure pump[2] was used in conjunction with

[1] Menrium, Hoffmann-La Roche, Nutley, N.J.

[2] Haskel pump in Dupont Instruments model 830 liquid chromatograph.


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**Table I—Linearity Range of Detector Response and Retention Times of Estrogen Compounds**

| Compound | Linear Range, μg injected | Retention Time, min | Relative Retention Time |
|---|---|---|---|
| 2,3,6-Trimethylphenol | — | 17.0 | 1.00 |
| Equilinen | 0.02–0.09 | 23.8 | 1.40 |
| Equilin | 0.09–0.45 | 26.4 | 1.55 |
| Estrone | 0.06–0.30 | 29.1 | 1.71 |
| Estradiol (17β-isomer) | 0.03–0.12 | 32.6 | 1.92 |

a septumless injector[3], a variable wavelength detector[4] set at 280 nm, and a 30-cm × 4-mm i.d. partition column containing silica permanently bonded with octadecylsilane[5].

**Reagents and Standards**—The mobile phase was methanol–water (60:40). Methanol and anhydrous sodium sulfate were reagent grade. The four estrogens used in the standard preparation were reference grade (99%+), as was the internal standard, 2,3,6-trimethylphenol.

**Chromatographic Separation**—The procedure was run at ambient temperature with a solvent flow of 0.6 ml/min. The UV monitor was set at 280 nm with a sensitivity of 0.04 absorbance unit. Standards and samples were injected with a 10-μl syringe.

**Standard Solution Preparations**—*Internal Standard*—Weigh accurately 20 mg of 2,3,6-trimethylphenol into a 100-ml volumetric flask. Dissolve and dilute to volume with methanol (internal standard stock solution).

*Estrone Reference Standard*—Weigh accurately 32 mg of reference standard into a 25-ml volumetric flask. Dissolve and dilute to volume with methanol.

*Equilin Reference Standard*—Weigh accurately 12.5 mg of reference standard into a 100-ml volumetric flask. Dissolve and dilute to volume with methanol.

*Equilenin Reference Standard*—Weigh accurately 12 mg of reference standard into a 25-ml volumetric flask. Dissolve and dilute to volume with methanol. Subdilute by pipetting 2 ml into a 25-ml volumetric flask and bring to volume with methanol.

*Estradiol Reference Standard*—Weigh accurately 10 mg of reference standard into a 25-ml volumetric flask. Dissolve and dilute to volume with methanol. Subdilute by pipetting 3 ml into a 25-ml volumetric flask and bring to volume with methanol.

**Combined Reference Standard Solution**—Into a 50-ml glass-stoppered erlenmeyer flask, pipet the following amounts of the previously prepared individual reference standards: 2 ml of estrone solution, 3 ml of equilin solution, 2 ml of subdiluted equilenin solution, and 3 ml of subdiluted estradiol solution.

Evaporate the methanolic mixture of estrogens to dryness under a nitrogen stream. To the dried residue, add 10 ml of the internal standard stock solution. Stopper the flask and mix thoroughly for complete dissolution (working standard solution).

**Sample Preparation**—Perform this preparation in duplicate. Into a 50-ml glass-stoppered centrifuge tube, accurately weigh a portion of either the bulk substance or finely ground tablet mass equivalent to 4 mg of esterified estrogens. Add 10 ml of 1 *N* HCl and swirl the tube contents until the powder is completely dispersed; then shake mechanically for approximately 5 min. After removal from the shaker, extract the acidified sample with five 20-ml portions of ether, shaking each extract mechanically for 5 min. (A gel-like emulsion is formed upon shaking but clears up immediately on standing; centrifuging hastens the process.) Withdraw the ether layer with a syringe, passing each extract through a layer of anhydrous sodium sulfate, and collect the combined extracts in an appropriately sized (~200 ml) amber, glass-stoppered erlenmeyer flask.

After the final extraction, rinse the sodium sulfate with additional ether, add to the combined extracts, and then evaporate to dryness with a nitrogen stream. Do not use heat! Dissolve the completely dried residue in 10 ml of the internal standard stock solution (working sample solution).

**Procedure**—Alternately inject 10 μl of the working standard and sample solutions into the liquid chromatograph to evaluate the retention times and condition of the column and to ensure constant response.

[3] Model U6K injector, Waters Associates, Milford, Mass.

[4] Model 770 spectroflow monitor, Schoeffel Instruments Corp., Westwood, N.J.

[5] μBondapak $C_{18}$, Waters Associates, Milford, Mass.

**Table II—Analytical Results for a Typical Lot of Esterified Estrogens Bulk Substance**

| Estrogen Quantitated | HPLC Assay | Colorimetric Assay[a] |
|---|---|---|
| Equilenin, mg/g | 0.83 | — |
| Equilin, mg/g | 5.11 | 3.20 |
| Estrone, mg/g | 28.88 | 28.28 |
| Estradiol (17β-isomer), mg/g | 0.72 | — |
| Total estrogen content, mg/g | 35.54 | 34.89 |
| Percent claim | 103 | 101.1 |
| Equilin to estrone ratio | 0.177 | 0.113 |

[a] Results as reported by the vendor *via* protocol.

Chromatograph duplicate standards and samples, and determine the respective responses using integration or peak height measurements.

**Calculations**—The response ratio for any particular estrogen, $RR_E$, may be determined as follows:

$$RR_E = \frac{P_E(\text{std})C_{IS}}{P_{IS}(\text{std})C_E} \qquad \text{(Eq. 1)}$$

where $P_E$ is the peak height of the estrogen in the working standard, $P_{IS}$ is the peak height of the internal standard in the working standard, $C_{IS}$ is the concentration of the internal standard (milligrams per milliliter) in the working standard, and $C_E$ is the concentration of the estrogen (milligrams per milliliter) in the working standard.

The amount of each esterified estrogen per gram of sample may be calculated as follows:

$$\text{mg of esterified estrogen/g of sample} = \frac{P_E(\text{spl})C_{IS}F}{P_{IS}(\text{spl})RR_E \text{ sample weight (g)}} \qquad \text{(Eq. 2)}$$

where $P_E$ is the peak height of the estrogen in the working sample, $P_{IS}$ is the peak height of the internal standard in the working sample, $RR_E$ is the response ratio for the estrogen as described, $C_{IS}$ is the concentration of the internal standard (milligrams per milliliter) in the working sample, and $F$ is the dilution factor multiplied by the factor for converting free estrogen to the esterified estrogen = 10 × 1.38[6].

The amount of each esterified estrogen per tablet may be calculated by multiplying the milligrams of esterified estrogen per gram by the average unit weight.

After each component has been quantitated, the total esterified estrogen content of a bulk powder or tablet formulation may be determined by adding the amounts found for equilenin, equilin, estrone, and estradiol.

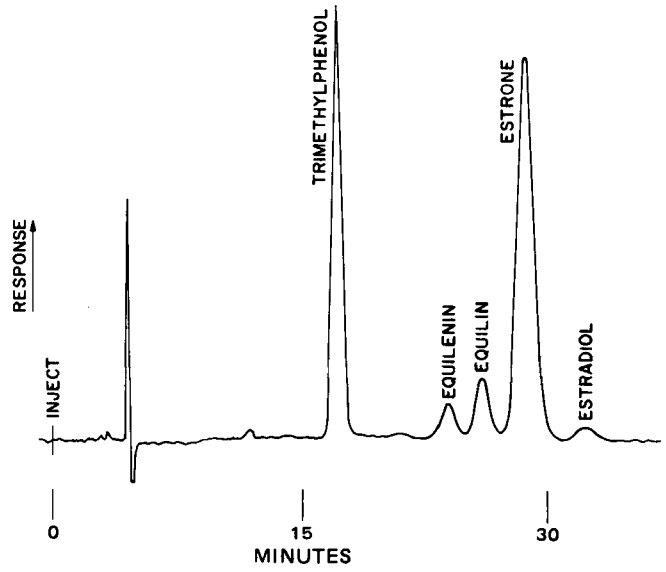


**Figure 1**—*Typical standard chromatogram for estrogen and internal standard compounds.*

[6] The molecular weight of sodium salt of the esterified estrogen divided by the molecular weight of the free estrogen is 1.38.

**Table III—Estrogen Content (Milligrams per Tablet) of Various Sample Lots by HPLC and the Spectrophotometric Procedure**

| Lot | Equilenin | Equilin | Estrone | Estradiol | Total[a] Equilin + Estrone | Total Four Estrogens | Spectro-photometric[b] |
|---|---|---|---|---|---|---|---|
| A-1 | 0.005 | 0.035 | 0.172 | 0.003 | 0.207 | 0.215 | 0.197 |
| A-2 | 0.004 | 0.032 | 0.167 | 0.004 | 0.199 | 0.206 | 0.206 |
| A-3 | 0.005 | 0.035 | 0.176 | 0.005 | 0.211 | 0.221 | 0.208 |
| A-4 | 0.005 | 0.038 | 0.179 | 0.004 | 0.217 | 0.226 | 0.197 |
| A-5 | 0.005 | 0.038 | 0.178 | 0.004 | 0.216 | 0.225 | 0.196 |
| B-1 | 0.010 | 0.072 | 0.356 | 0.007 | 0.427 | 0.444 | 0.419 |
| B-2 | 0.010 | 0.072 | 0.352 | 0.009 | 0.424 | 0.442 | 0.404 |
| C-K[c]-1 | 0.011 | 0.070 | 0.332 | 0.010 | 0.402 | 0.412 | 0.417 |
| C-C[d]-1 | 0.012 | 0.057 | 0.316 | 0.008 | 0.373 | 0.392 | 0.411 |
| C-K[c]-2 | 0.011 | 0.050 | 0.324 | 0.009 | 0.374 | 0.408 | 0.406 |
| C-C[d]-2 | 0.013 | 0.062 | 0.329 | 0.010 | 0.391 | 0.414 | 0.391 |

[a] HPLC determination. [b] Equilin + estrone content. [c] Kernels or uncoated tablets. [d] Coated tablets.

## RESULTS AND DISCUSSION

The determination of esterified estrogens in bulk substrates and combination dosage tablets presents many analytical problems. The time-consuming sample preparation prior to the colorimetric assay has too many steps where errors can be introduced, making final results questionable.

After a simple hydrolysis procedure, the converted free estrogens are extracted readily with ether. The evaporated extract residues, reconstituted in a methanolic solution containing the internal standard, are chromatographed; a run is completed in approximately 35 min. Typical standard and sample chromatograms are shown in Figs. 1 and 2.

With a flow rate of 0.6 ml/min, theoretical plates were calculated to range between 2100 and 3700 for the estrogens. Linearity of detector response and relative retention time data of each quantifiable estrogen are listed in Table I.

To verify noninterference of tablet excipients with estrogen elutions, placebo tablets were pulverized, extracted, reconstituted in methanol, and chromatographed. A baseline run was obtained, clearly showing no interference. Another portion of the powdered placebo mass was spiked with an equivalent amount of the bulk sodium estrone sulfate; upon extraction and chromatography, the recovery of 99+% was achieved.

A mixture of chlordiazepoxide base and ground placebo tablets, in the proportion found in the particular dosage form, was subjected to the extraction procedure and chromatography; no extraneous peaks were evident in any areas of the estrogen elutions.

To assure completeness of the extraction procedure, five individual extracts of 20 ml of ether were evaporated to dryness, dissolved in 2 ml of methanol, and chromatographed. This procedure was performed on two lots of tablets and on a bulk lot of the esterified estrogen mixture. The component percentages found per individual estrogens averaged 45.5 and 43.8 for the first and second extractions, respectively. The remaining extracts contained 5–20% for the third, 2–6% for the fourth, and 1% for the fifth. These data substantiated the need for more than three extractions.

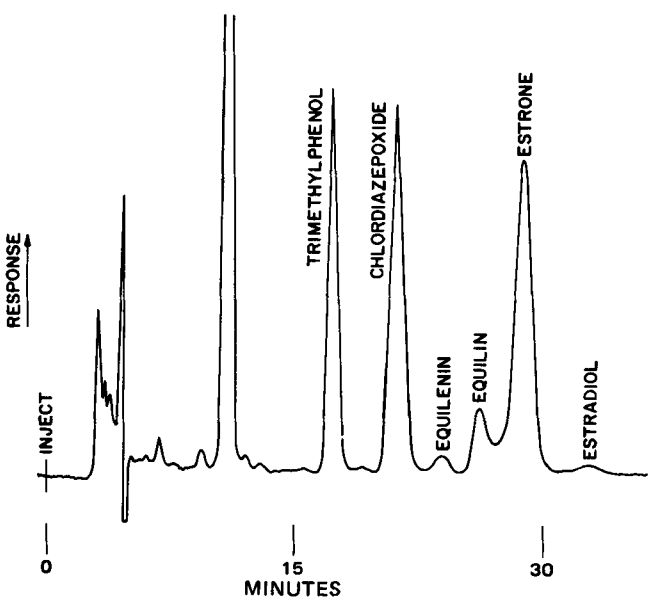


**Figure 2**—*Typical sample chromatogram for a tablet formulation.*

Results obtained for a typical lot of esterified estrogen bulk substance using the proposed HPLC method and the current spectrophotometric procedure are presented in Table II; data obtained from analyses of various dosage forms are compiled in Table III.

Statistical evaluations performed on six samples per lot of uncoated and coated tablets produced the following results (milligrams per tablet) for total estrogens: Lot C-K-1, 0.442, 0.429, 0.380, 0.390, and 0.423 (mean ± $SD$ = 0.41 ± 0.02); Lot C-C-1, 0.375, 0.380, 0.379, 0.391, 0.396, and 0.411 (mean ± $SD$ = 0.39 ± 0.02); Lot C-K-2, 0.391, 0.398, 0.411, 0.418, 0.412, and 0.418 (mean ± $SD$ = 0.42 ± 0.01); and Lot C-C-2, 0.405, 0.414, 0.445, 0.398, 0.411, and 0.408 (mean ± $SD$ = 0.41 ± 0.02).

The data presented affirm that this method is more acceptable than the procedure currently used because it is a rapid analytical technique of high precision and specificity.

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# Surface Activities of Procaine, Lidocaine, and Tetracaine and Their Interaction Energies with Phospholipid Monolayers

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**Abstract** □ The free energies of adsorption of procaine, lidocaine, and tetracaine at the air–water interface were estimated from plots of surface pressure ($\pi \leq 5$ dynes/cm) against bulk concentration. Their interaction energies with dipalmitoylphosphatidylethanolamine and dipalmitoyllecithin monolayers, previously spread at the air–water interface, were estimated from the increase of surface pressure with increasing concentrations of the subphase-injected anesthetic. Free energies of adsorption and the interaction energies were in the order procaine < lidocaine < tetracaine and correlate with relative anesthetic potencies and the blocking of nerve conduction.

**Keyphrases** □ Anesthetics, local—free energies of adsorption at air–water interface and interaction energies with phospholipid monolayers correlated with pharmacological activity □ Adsorption, free energies—various local anesthetics at air–water interface, correlated with pharmacological activity □ Interaction energies—various local anesthetics with phospholipid monolayers, correlated with pharmacological activity □ Phospholipid monolayers—interaction energies with various local anesthetics correlated with pharmacological activity □ Surface activities—various local anesthetics, correlated with pharmacological activity

The correlation between the potency of local anesthetics in blocking nerve conduction and their penetrations into lipidic monolayers has been demonstrated (1–5). More recently, it was shown by NMR (6, 7) and spin-labeled local anesthetics (8) that they do penetrate into zwitterionic phospholipid bilayers of liposomes.

The present work examines the surface activities of procaine, lidocaine, and tetracaine at the air–aqueous interface and estimates their interaction energies with dipalmitoylphosphatidylethanolamine and dipalmitoyllecithin monolayers, previously spread at the air–aqueous interface.

## EXPERIMENTAL

**Reagents**—Procaine hydrochloride[1], lidocaine hydrochloride[2], and tetracaine hydrochloride[2] were used without further purification. Dipalmitoyllecithin[3], dipalmitoylphosphatidylethanolamine[4], hexane[5] used for the preparation of the spreading solutions, and distilled water used for the preparation of the solutions fulfilled the requirements previously specified (9, 10). Analytical reagent sodium chloride[6] was roasted for 6 hr at 700° prior to the preparation of the aqueous solutions to remove surface-active organic impurities.

**Instruments and Methods**—Surface tension was measured with a Wilhelmy platinum plate attached to an electrobalance[7] whose output was fed into a recorder[8]. The methods for the measurement of the surface tension of aqueous solutions, $\gamma$, and the change of the surface pressure, $\Delta\pi$, of the phospholipid monolayer as a function of time after drug injection in the subphase were described previously (9–11). All experiments were performed in 0.15 *M* NaCl at 20 ± 1°.

In the injection experiments, the initial surface pressure of the phospholipid monolayer spread at the air–0.15 *M* NaCl interface was 5 ± 0.1 dynes/cm. The surface pressure, $\pi$, of the drug solution is the difference between the previously determined surface tension of the 0.15 *M* NaCl, $\gamma_{NaCl}$, and the surface tension of the drug solution in this saline solution, $\gamma_d$. The surface pressure, $\pi$, was fitted to a function of the drug concentration, *C*, by digital computerized nonlinear regression to exponential equations of the form:

$$\pi = \gamma_{NaCl} - \gamma_d = B_1 e^{[B_2(\log C)^2 + B_3 \log C]} \qquad \text{(Eq. 1)}$$

where the $B_i$ values were adjustable parameters (9).

Densities of the drug solutions were determined with 10-ml specific gravity bottles previously calibrated with water. The precision of the weighing was ± 0.1 mg.

## RESULTS

**Adsorption at Air–Aqueous Interface**—Typical plots of the surface pressure against the logarithm of the concentration (moles per liter) for procaine hydrochloride, lidocaine hydrochloride, and tetracaine hydrochloride in 0.15 *M* NaCl are given in Fig. 1.

A simple expression for the free energy of adsorption was derived (12) from thermodynamic and molecular kinetic considerations:

$$\Delta G^\circ = -RT \ln \frac{\pi}{X_2^*} \qquad \text{(Eq. 2)}$$

where $\Delta G^\circ$ is the change in standard free energy associated with the adsorption of the solute at an air–water interface, $\pi$ is the surface pressure, $X_2^*$ is the activity of the solute, and $R$ and $T$ are the universal gas constant and the absolute temperature, respectively. Thus, the numerical value of $\Delta G$ can be calculated from the slope, $\pi/X_2^*$, of a linear plot of $\pi$ against the mole fraction, $X_2$, of the solute in bulk solution when $X_2 \to 0$ and $X_2 \to X_2^*$ at low mole fractions. This expression was used recently for the estimation of the free energy of adsorption of alkanols from $C_1$ to $C_{14}$ at the air–aqueous interface (11).

The plots (Fig. 2) of $\pi$ against $X_2$ for the three compounds were reasonably linear in the region $\pi \leq 5$ dynes/cm under the experimental

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Pfaltz and Bauer, Stamford, Conn.
[3] Applied Science Laboratories, State College, Pa.
[4] Schwarz-Mann Research Laboratories, Orangeburg, N.Y.
[5] J. H. Baker Chemical Co., Phillipsburg, N.J.
[6] Mallinckrodt Chemical Works, St. Louis, Mo.
[7] Cahn Division, Ventron Instrumental Corp., Paramount, Calif.
[8] Leeds Northrup, North Wales, Pa.



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