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Note

Quantitative analysis of ethisterone and ethynyl oestradiol preparations by high-performance liquid chromatography

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Preparations containing a mixture of ethisterone and ethynyl oestradiol, in a ratio of 1000:1, are used in the symptomatic treatment of secondary amenorrhea where pregnancy has been ruled out as the cause. Such a high ratio makes the simultaneous analysis of the two active ingredients very difficult.

Ethisterone exhibits a good UV absorption with maximum at *ca.* 240 nm[1], and is easily detected at this wavelength.

Ethynyl oestradiol has been previously detected using UV absorption at 280 nm[2,3] and 210 nm[4]. In order to increase its detectability several authors have described pre-column derivatization[5–7] followed by UV or fluorescence detection. Recently, the native fluorescence of the phenolic ring of oestrogens has been used for detection of ethynyl oestradiol in a high-performance liquid chromatographic (HPLC) procedure[8]. An electrochemical detector has also been found to be very sensitive and specific for compounds having a free phenolic group[9,10].

A reversed-phase HPLC system with UV and electrochemical detection is described for the simultaneous determination of ethisterone and ethynyl oestradiol in single dosage forms.

EXPERIMENTAL

Apparatus

A modular HPLC system was used, consisting of a solvent delivery system (SP 8700, Spectro-Physics, Santa Clara, CA, U.S.A.) operated at 1.0 ml/min, a variable-wavelength UV detector (Schoeffel Model SF 700, Westwood, NJ, U.S.A.) set at 270 nm, an electrochemical detector (Model LC-3, Bioanalytical System, West Lafayette, IN, U.S.A.) with the oxidation potential set at +900 mV, and a loop injector (Rheodyne septumless valve injector, Model 7120, Berkeley, CA, U.S.A.) equipped with a 20-μl loop. The column was a 300 × 4 mm I.D. commercially available octadecyl silane, chemically bonded to totally porous 10-μm micro-silica particles (Micropak MCH-10, Varian, Palo Alto, CA, U.S.A.).

Peak retention times and areas were obtained by the use of a reporting integrator (automation system 3385A, Hewlett-Packard, Avondale, PA, U.S.A.), or peak heights were measured manually from the chromatograms.

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Reagents

Ethynyl oestradiol was USP reference standard, and ethisterone and benzestrol were National Formulary reference standards. Biphenyl was purchased from Aldrich (Milwaukee, WI, U.S.A.). Acetonitrile was HPLC grade (Fisher, Fair Lawn, NJ, U.S.A.). Water was double-distilled in glass, and other reagents were analytical grade.

Mobile phase

Previously filtered and degassed acetonitrile and 0.01 *M* aqueous sodium formate solution were directly mixed by the solvent delivery system in a proportion of 55:45.

Internal standard solution

A solution containing benzestrol (5 μg/ml) and biphenyl (250 μg/ml) was prepared in 80% aqueous tetrahydrofuran.

Standard preparation

A solution containing ethynyl oestradiol (5 μg/ml) and ethisterone (5 mg/ml) was prepared in internal standard solution.

Sample preparation

A portion, accurately weighed, of finely powdered tablets, equivalent to *ca.* 50 mg ethisterone (one tablet), was transferred to a PTFE-lined screw-capped 15-ml culture tube. Exactly 10.0 ml of internal standard solution was added. The tube was capped and shaken for 30 min on a vortex-type mixer and then centrifuged.

Procedure

A 20-μl portion of the standard preparation and the sample preparation was successively injected into the column via the 20-μl loop.

The ratio (R) of the area of the ethisterone to the area of biphenyl (internal standard) was calculated for the standard preparation and sample preparation from the results obtained from the UV detector, and the ratio (R) of the peak height of ethynyl oestradiol to the peak height of benzestrol (internal standard) from the results obtained for the standard and sample preparation from the electrochemical detector.

The concentration of active ingredients in the sample preparation was obtained by the following formula:

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

where C_u = concentration of ethisterone or ethynyl oestradiol in sample preparation; C_s = concentration of ethisterone or ethynyl oestradiol in standard preparation; R_u = ratio of ethisterone peak area to biphenyl peak area, or ratio of ethynyl oestradiol peak height to benzestrol peak height for sample preparation; R_s = ratio of ethisterone peak area to biphenyl peak area or ratio of ethynyl oestradiol peak height to benzestrol peak height for the standard preparation.

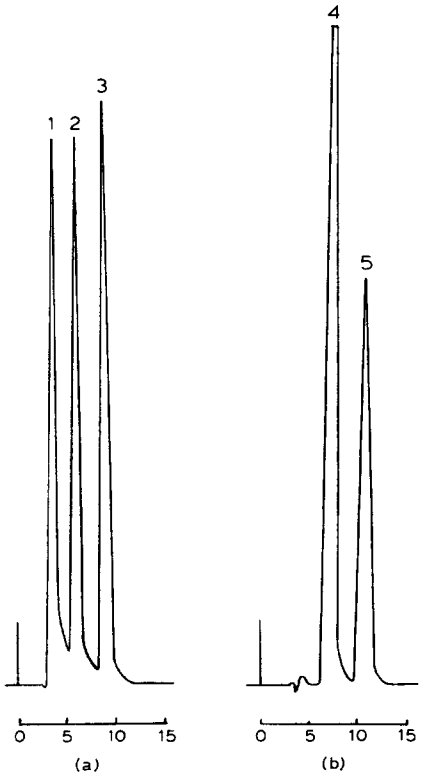

Fig. 1. HPLC chromatogram of tablet extract containing ethynyl oestradiol and ethisterone. (a) Electrochemical detector. Peaks: 1 = solvent; 2 = ethynyl oestradiol; 3 = benzestrol. (b) Ultraviolet detector. Peaks: 4 = ethisterone; 5 = biphenyl.

RESULTS AND DISCUSSION

Specific and sensitive detection of the free phenolic groups was obtained with the electrochemical detector, since only ethynyl oestradiol and benzestrol were detected. The ionisation (oxidation) potential was set at +900 mV, in order to obtain the optimum relationship between response factor and baseline noise. At this setting the minimum amount of each compound detected was 2 ng, linear response *versus* concentration was obtained up to 600 ng and ethisterone gave no detectable response.

TABLE I

RETENTION TIMES OF COMPOUNDS OF INTEREST

Name	t_R *(min)*	*Detector**
Ethynyl oestradiol	6.3	ED
Ethisterone	7.5	UV
Benzestrol	12.4	ED
Biphenyl	9.8	UV

* ED = electrochemical detector.

Within this range, the standard curve passed close to the origin and its correlation coefficient was nearly ideal (0.9999).

Sodium formate was added to the mobile phase to increase the sensitivity of the electrochemical detector. The sensitivity increased with ionic strength (buffer concentration), and sufficient sensitivity was found when 0.01 *M* sodium formate was used.

Ethisterone was detected at 270 nm even though its absorption maximum is at 240 nm. This wavelength provided a better linear response *versus* concentration without affecting too much the sensitivity. In the range studied (20–120 μg injected) there was found to be a linear response against concentration (correlation coefficient = 0.9992).

Baseline resolution was not achieved between ethisterone and ethynyl oestradiol (Fig. 1), but this was not a requirement as their respective modes of detection were quite independent of each other. The small UV contribution of ethynyl oestradiol to ethisterone is estimated at less than 0.1%.

Retention times of compounds of interest are listed in Table I.

The accuracy of the procedure was ascertained via a synthetic preparation. Recovery from this preparation for ethisterone was 100.2% with a standard deviation of 1.0% and for ethynyl oestradiol was 101.3% with a standard deviation of 0.8%.

Analysis of a commercial formulation gave the following results from a composite grind: ethisterone, 98.4% (SD 1.1%); ethynyl oestradiol, 124.8% (SD 4.5%).

These results show a problem with the ethynyl oestradiol content of the tablets. This was confirmed by a single-tablet analysis of ten tablets, which gave a range of results for ethynyl oestradiol of 60.1–152.2%; the ethisterone content was satisfactory for all ten tablets.

In spite of the apparent content uniformity problem with this batch of tablets, the procedure as described is satisfactory for the analysis of this particular mixture.

CONCLUSION

This HPLC procedure, using electrochemical and UV detection, is fast, accurate and is sufficiently sensitive to allow for single dosage form analysis when necessary.

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