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Note

Thin-layer chromatographic system for the semi-quantitative analysis of the ratio of α- and β-dihydroequilin in the diol fraction of equine estrogen conjugates

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Our earlier work[1,2] on the thin-layer chromatographic (TLC) separation of equine estrogens concentrated on the resolution of six components (estrone, equilin, equilenin and their respective 17α-diols). These systems were, of necessity, compromises which did not allow optimum resolution of either the ketones or the diols. When the need arose to isolate 17β-dihydroequilin from all other components, and to determine its amount relative to that of 17α-dihydroequilin, it was felt that a system more suitable than the earlier ones could be developed, based on the diol fraction alone, thus removing the constraint caused by the presence of the ketones.

EXPERIMENTAL

Plate preparation

Silica gel plates (0.25 mm, 20 × 20 cm) were prepared from a slurry of 30 g silica gel N/UV_{254} for TLC (Macherey, Nagel U Co., Düren, G.F.R.) with 70 ml of 1 *N* sodium hydroxide solution. The plates were allowed to dry overnight.

Plate impregnation

Plates were impregnated with formamide by immersing in a 20% solution of formamide in acetone for 5 min. Samples were applied to the plates as soon as the acetone had evaporated.

Solvent system

The solvent system used was xylene–diethylamine (200:1). Tanks were lined with Whatman No. 1 filter paper and allowed to equilibrate for 1 h before use.

Standard solution

A solution containing 1 mg/ml of 17β-estradiol in methylene chloride was prepared.

Diol fraction preparation

A quantity of finely powdered tablets containing an equine estrogen extract

accurately weighed and equivalent to about 10 mg of equine estrogens was mixed with 8 g of chromatographic siliceous earth. A 3-ml volume of water was added, with mixing. A 3-g amount of chromatographic siliceous earth was added to a 175 × 25 mm chromatographic tube, after which the conjugated estrogens mixture was added. With the aid of reduced pressure, the column was washed with 100 ml of benzene (flow-rate 3–5 ml/min). The column was then eluted with 150 ml of dicyclohexylamine acetate solution (225 mg in 150 ml of chloroform–water-saturated diethyl ether, 60:40). The flow-rate was maintained at 3–5 ml/min. The eluate was collected under reduced pressure and evaporated cautiously on a rotary evaporator until the residue was almost dry. About 10 ml of methanol was added to the residue, and the solution was then evaporated to dryness. The residue was dissolved in 20 ml of methanol, a few anti-bumping granules being added. A 6.0-ml volume of 5% (v/v) hydrochloric acid was added, and the solution was refluxed for 12.0 min after which the flask was cooled in an ice-bath. The solution was transferred to a 250-ml separator with the aid of 60 ml of 6.5% (w/v) aqueous potassium hydroxide and mixed. The solution was washed with two 60-ml portions of carbon tetrachloride, and the washings were combined and extracted with a 40 ml portion of aqueous potassium hydroxide solution. The extract was added to the washed alkaline solution. Sufficient 33% (v/v) sulfuric acid was added to adjust to pH 1. The acidified solution was extracted immediately with a 20-ml and then a 15-ml portion of benzene, shaking vigorously each time for 1 min. The benzene extracts were combined and washed wit ha 10-ml portion of water, which was then backwashed with a 10-ml portion of benzene. The benzene extracts were combined.

A chromatographic column was prepared by mixing 3 g of chromatographic siliceous earth with 1 ml of 10% (w/v) sodium carbonate solution. This mixture was transferred to a 175 × 25 mm chromatographic tube. The benzene extracts were passed through the chromatographic column, and filtered through 3–4 g of anhydrous sodium sulfate and the eluate was collected in a 100-ml volumetric flask. All apparatus was washed with several 10-ml portions of benzene, adding each portion to the 100-ml volumetric flask. Benzene was added to volume.

A 30.0 ml volume of the diluted benzene solution was transferred to a 50-ml conical flask, and carefully evaporated to dryness with the aid of gentle heat and a stream of nitrogen so that all of the residue was deposited on the bottom of the flask. A further 30.0 ml was added to the flask and similarly treated. A 100-mg amount of trimethylacethydrazide ammonium chloride and 0.5 ml of glacial acetic acid were added to the residue. The flask was covered with a watch glass, and heated on a steam bath for 5 min. with occasional swirling, to assure complete conversion. The solution was cooled and transferred with the aid of 50 ml of ice-cold water to a 125-ml separator containing 10 ml of 5% (w/v) aqueous sodium acetate solution. The solution was extracted rapidly with four 10-ml portions of chloroform, and the extracts were combined in a second separator and washed with 5 ml of water. The chloroform solution was filtered through a pledget of glass-wool upon which 10 g of anhydrous sodium sulfate had been placed, the filtrate being collected in a 100-ml round-bottom flask and evaporated to dryness on a rotary evaporator. The residue was dissolved in 4 ml of methylene chloride.

TLC procedure

Portions of 1, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 μl of the above sample preparation and a 10 μl portion of standard 17β-estradiol preparation (1 mg/ml) were applied along the origin of freshly impregnated plates, taking care to form a spot not greater than about 5 mm in diameter. Solutions could not be applied closer than 3 cm to either edge of the plate because of a significant edge effect. Plates were developed for 15 cm after which they were removed from the tank and allowed to dry. Plates were sprayed with a freshly prepared 0.5% aqueous solution of Fast Blue B salt[3] until the spots were very prominent, then the plates were heated at 105° for 20 min, after which time the brown spots of the dihydroequilins had turned a distinctive purple (relative R_F values and colors of spots are shown in Table I).

TABLE I

RELATIVE R_F VALUES AND COLORS OF SPOTS

17β-Estradiol = 1.00.

Compound	*Relative R_F*	*Color*
17α-Estradiol	1.16	Yellow
17β-Estradiol	1.00	Yellow
17α-Dihydroequilin	0.99	Purple
17β-Dihydroequilin	0.79	Purple
17α-Dihydroequilenin	0.65	Blue
17β-Dihydroequilenin	0.50	Blue

Equivalence of response

The equivalence of responses of 17α- and 17β-dihydroequilin to the color development procedure was determined by stepwise application of different volumes of standard solutions of equal concentration to TLC plates, followed by chromatography as described above.

Ratio of 17α- to 17β-dihydroequilin

From the developed plates the minimum applied volumes of sample solution required to distinguish the spots of 17α-dihydroequilin and 17β-dihydroequilin were determined. Thus, the ratio of 17α-dihydroequilin to 17β-dihydroequilin was determined from:

$$\text{ratio} = \frac{\text{minimum applied volume for 17}\alpha\text{-dihydroequilin}}{\text{minimum applied volume for 17}\beta\text{-dihydroequilin}}$$

DISCUSSION

The 17β-estradiol is used as a reference standard in order to assist in the location of the 17α-dihydroequilin spot. Although there may be a trace amount of 17β-estradiol some of the samples, this does not interfere with the procedure for determining the ratio of 17α- to 17β-dihydroequilin, because 17α-dihydroequilin is the most abundant component of the diol fraction, and at its minimum applied volume in the procedure, it is the only component visible.

Results obtained from some typical samples range from a α/β ratio of 8 up to 20, and in some cases even higher, where no 17β-dihydroequilin could be seen in a 20-μl portion.

CONCLUSION

A TLC procedure has been developed for the determination of the ratio of 17α- to 17β-dihydroequilin in the diol fraction of equine estrogen conjugates.

REFERENCES

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