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Short Communication

Rapid high-performance liquid chromatographic assay of ethynyloestradiol in rabbit plasma

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ABSTRACT

A method for the determination of ethynyloestradiol in samples of rabbit plasma containing pentobarbital and heparin, the former used as an anaesthetic and the latter as an anticoagulant, has been developed. Quantification was carried out using a reversed-phase high-performance liquid chromatographic (HPLC) method in isocratic mode at room temperature, with electrochemical detection at an applied potential of +1 V *vs.* Ag/AgCl. Under these conditions, the retention time for ethynyloestradiol was *ca.* 2.9 min, the average recovery from plasma was 74.5%, and the limit of detection was 10 pg, corresponding to a plasma concentration of 50 pg/ml using 1 ml of plasma. Natural oestrogens, oestriol, oestradiol and oestrone showed peaks that did not interfere with ethynyloestradiol, and retention times of *ca.* 0.8, 2.4 and 3.4 min, respectively.

INTRODUCTION

The semisynthetic oestrogen ethynyloestradiol is widely used as a component of the oral contraceptive pill, the usual daily dose being only 30–50 μg. Thus in order to measure plasma concentrations a sensitive and accurate assay method is required.

Radioimmunoassay (RIA) is generally used at present to measure very low levels [1–6], but the treatment is slow and complex and it also shows cross-reactivity with several natural oestrogens [4]. We have also found several methods to determine ethynyloestradiol by high-performance liquid chromatography (HPLC) in non-biological samples with UV [7–10] and fluorescence [11] detection, but minimum detection levels are too high for present requirements. From the paper by Suzuki *et al.* [12], which describes an automated system for the direct measurement of sex steroid hormones in serum using HPLC with electrochemical detection, we have developed a new method to detect low levels of ethynyloestradiol in rabbit plasma containing pentobarbital and treated with heparin as anticoagulant. Pentobarbital was used to anaesthetize rabbits with the aim of extracting blood samples frequently from the carotid artery at short periods of time. Our method also shows a remarkable reduction in analysis time compared with the work by Suzuki *et al.* [12]. This technique is superior in simplicity, rapidity and safety to RIA, which is very important in clinical practice.

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EXPERIMENTAL

Reagents

Ethynyloestradiol (Sigma, St. Louis, MO, USA), oestriol (Sigma), oestradiol (Sigma), oestrone (Sigma), sodium pentobarbital (Barcia, Madrid, Spain), sodium heparin 1% solution (Rovi, Madrid, Spain), LC-grade water obtained in a Milli-Q system (Millipore, Bedford, MA, USA), HPLC-grade diethyl ether (Romil, Loughborough, UK), HPLC-grade methanol (Merck, Darmstadt, Germany), HPLC-grade acetonitrile (Panreac, Barcelona, Spain), analytical-grade sodium hydroxide (Panreac), analytical-grade potassium dihydrogenphosphate (Merck), and analytical-grade phosphoric acid (Panreac) were used.

Apparatus

The HPLC system consisted of a Model Isochrom pump (Spectra-Physics, San Jose, CA, USA), a pulsed electrochemical detector (Model 464, Waters, Milford, MA, USA), a computing integrator (Model SP4400, Spectra-Physics), a sample injector fitted with a 50-μl loop (Model Rheodyne 7125, Rheodyne, Cotati, CA, USA), and a Novapack C_{18} 4-μm reversed-phase cartridge (150 mm × 3.9 mm I.D, Waters). A slow rotatory mixer (Selecta, Barcelona, Spain) was used in extraction procedures.

Chromatographic conditions

A mobile phase of 50 m*M* potassium phosphate buffer (50 m*M* potassium dihydrogenphosphate solution adjusted to pH 3.6 with phosphoric acid)–acetonitrile–methanol (10:7:3, v/v/v) was used. The flow-rate was 1.6 ml/min, and the column effluents were monitored at an applied potential of +1 V *vs.* Ag/AgCl. The procedure was carried out at room temperature. Retention times were 2.94 min for ethynyloestradiol, 0.83 min for oestriol, 2.44 min for oestradiol, 3.44 min for oestrone and 0.99 min for heparin. Pentobarbital was not detected.

Preparations of standards

An ethynyloestradiol stock solution (1 μg/ml) in the mobile phase was prepared for HPLC calibration. The stock solution was diluted with the mobile phase in order to obtain nine standard solutions at 0.2, 0.4, 1, 2, 5, 10, 20, 50 and 100 ng/ml. For recovery experiments, five plasma standards of ethynyloestradiol were prepared by spiking a mixture of 1 ml of a drug-free pooled rabbit plasma with an aliquot of each standard solutions in the mobile phase at appropiate concentrations. A standard solution of ethynyloestradiol, oestriol, oestradiol and oestrone, containing 100 ng/ml of each in the mobile phase, was also prepared and treated in the same way (Fig. 1).

Extraction procedure

A 0.5-ml volume of 10 *M* NaOH was added to each 1-ml plasma standard solution, and then shaken on a slow rotatory mixer for 5 min. The mixture was extracted with 5 ml of diethyl ether by rotomixing for 10 min. After centrifugation for 5 min at 700 *g*, the organic layer was removed by aspiration and transferred to a glass tube. The extraction procedure was repeated with an other 5 ml of diethyl ether. The total organic layer was evaporated to dryness under a stream of nitrogen at 37°C. The residue was redissolved in 250 μl of the mobile phase, and two portions were withdrawn to rinse and load the 50-μl loop for injection.

Quantification

Four injections of each standard solution were made up. The peak heights of standard solutions were analysed by linear regression with respect to their concentrations.

RESULTS AND DISCUSSION

The regression equations calculated for calibration, with corresponding correlation coefficients (r^2) over the ranges studied, were: $y = 826.6511 + 2331.9470x$, $r^2 = 0.9919$ for the range 0.2–5 ng/ml and $y = -2326.7508 + 3045.6149x$, $r^2 = 0.9990$ for the range 5–100 ng/ml. It was necessary to use these two concentration ranges to obtain a better correlation ($p <$

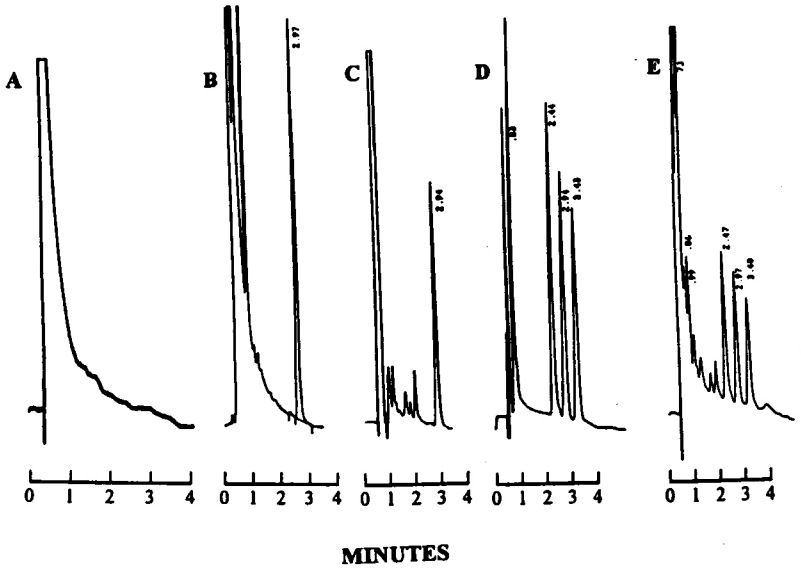

Fig. 1. Chromatograms obtained from (A) blank plasma after extraction (0.032 a.u.f.s.), (B) ethynyloestradiol in standard solution (5 ng, 0.256 a.u.f.s., t_R = 2.97 min), (C) 1 ml of rabbit plasma spiked with 10 ng of ethynyloestradiol and extracted using our method (0.256 a.u.f.s., t_R = 2.94 min), (D) oestriol (5 ng, t_R = 0.83 min), oestradiol (5 ng, t_R = 2.44 min), ethynyloestradiol (5 ng, t_R = 2.94 min) and oestrone (5 ng, t_R = 3.43 min) in standard solution (0.256 a.u.f.s.) and (E) 1 ml of rabbit plasma spiked with oestriol (10 ng, t_R = 0.80 min), oestradiol (10 ng, t_R = 2.47 min), ethynyloestradiol (10 ng, t_R = 2.97 min) and oestrone (10 ng, t_R = 3.48 min) extracted using our method (0.256 a.u.f.s.). For conditions see text.

0.001). Fig. 1A shows a chromatogram of a blank plasma sample after extraction. Fig. 1B shows a chromatogram of the standard solution containing ethynyloestradiol, and Fig. 1C is a chromatogram of extracted rabbit plasma spiked with ethynyloestradiol. Chromatograms of the standard solution containing oestriol, oestradiol, ethynyloestradiol and oestrone (Fig. 1D) and the same solution after extraction from spiked rabbit plasma (Fig. 1E) are also shown.

TABLE I

INTER-DAY PRECISION AND ACCURACY FOR THE ASSAY OF ETHYNYLOESTRADIOL IN THE STANDARD SOLUTION

Ethynyloestradiol standard solution (ng/ml)	Number of samples	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
0.2	8	0.21 ± 0.02	11.12
0.4	11	0.41 ± 0.04	10.85
1	10	1.04 ± 0.05	5.21
2	11	1.95 ± 0.09	4.66
5	14	4.91 ± 0.19	2.68
10	12	10.51 ± 0.48	4.53
20	13	21.06 ± 0.79	3.74
50	8	49.24 ± 1.11	2.26
100	12	100.05 ± 5.68	5.69

TABLE II

INTER-DAY PRECISION AND ACCURACY FOR ETHYNYLOESTRADIOL IN PLASMA, AND RECOVERIES FROM PLASMA

Ethynyloestradiol concentration spiked in plasma (ng/ml)	Number of samples	Concentration found (ng/ml)		Coefficient of variation (%)	Recovery (%)
		Individual values	Mean ± S.D.		
0.1	7	0.063	0.074 ± 0.010	13.831	62.50
		0.069			69.00
		0.086			86.00
		0.083			83.25
		0.061			61.00
		0.073			72.93
		0.082			82.32
0.5	7	0.335	0.374 ± 0.055	14.723	67.00
		0.440			88.00
		0.360			72.00
		0.348			69.50
		0.297			59.48
		0.447			89.32
		0.390			78.05
1	7	0.755	0.771 ± 0.060	7.771	75.50
		0.768			76.75
		0.703			70.25
		0.886			88.55
		0.796			79.58
		0.774			77.43
		0.718			71.76
5	8	4.108	3.927 ± 0.407	10.371	82.15
		4.675			93.50
		3.500			70.00
		4.113			82.26
		3.643			72.85
		3.968			79.37
		3.413			68.22
		3.997			79.94
25	6	14.708	16.700 ± 2.450	14.670	58.83
		20.655			82.62
		18.515			74.06
		14.200			56.20
		16.199			64.80
		15.923			63.69
				Mean ± S.D. =	74.53 ± 9.51

The minimum calculable concentration of ethynyloestradiol in plasma was 50 pg/ml, but it would be possible to detect lower concentrations using, for example, a greater volume of plasma, injecting a greater volume of solution, or redissolving it in a smaller volume of mobile phase. Data on the inter-day precision and accuracy of the method for ethynyloestradiol in the standard solutions are shown in Table I. The average recovery of ethynylestradiol from the standard solutions was 74.53% (Table II). Data on the calculation of the inter-day precision in the standard solutions in plasma are also shown in Table II.

This method allows rapid and easy determina-

tion of ethynyloestradiol in rabbit plasma samples in the presence of heparin and pentobarbital, and even if the natural oestrogens, oestriol, oestradiol and oestrone were present they could be clearly separated from ethynyloestradiol.

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