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Development of a highly sensitive and specific assay for plasma ethinylestradiol using combined extraction, liquid chromatography and radioimmunoassay

RICHARD L. TACEY,* WARREN J. HARMAN and LAURA L. KELLY

Chemistry Department, Hazleton Washington, Inc., Vienna, VA 22182-1699, USA

Abstract: A highly sensitive and specific assay for ethinylestradiol (EE2) in human plasma was developed. The assay procedure combined solid-phase extraction of plasma samples, isolation of extracted EE2 by liquid chromatography (LC), and radioimmunoassay. Samples were extracted to remove polar plasma constituents and steroid binding proteins. Chromatography was employed to separate EE2 from other steroids that were candidates for assay cross-reactivity. The radioimmunoassay was shown to be sensitive (lower limit of quantitation = 2 pg ml^{-1} EE2 in plasma) and accurate (mean accuracy = 102%). Recovery of EE2 through extraction and LC steps was 76.1 ± 4.5% ($\bar{x}$ ± SD; n = 42). Overall assay intra- and inter-assay coefficients of variation were 3.6 and 8.9%, respectively. The analyte was stable in assay buffer and assay accuracy was influenced minimally by four sample freeze–thaw cycles. This assay protocol enables the precise monitoring of low circulating levels of EE2, a prominent and potent synthetic oestrogen.

Keywords: *Ethinylestradiol; ethinylestradiol; radioimmunoassay; liquid chromatography.*

Introduction

The oestrogen analogue ethinylestradiol (EE2; Fig. 1) is a widely used therapeutic agent. A component of most oral contraceptive formulations [1–3] used on a regular basis by 55–60 million women [4], EE2 has also been found useful in many applications for which a potent oestrogen was needed; in treatment regimens for hirsutism [5], prostate cancer [6], Turner's Syndrome [7], puberty induction in girls [8], prepubertal growth control in girls [9], and side-effects of menopause [10]. Furthermore, EE2 in combination with other (usually progestogenic) steroids has proven to be beneficial therapy for polycystic ovary syndrome [11], primary dysmenorrhea [12], and as a postcoital contraceptive [13].

Concerns of adverse side effects from EE2 [1, 6, 14] have led to efforts to reduce EE2 therapeutic concentrations to the lowest effective levels, while co-administration with drugs such as antibiotics, enzyme-inducing agents, ascorbic acid or paraminophen may

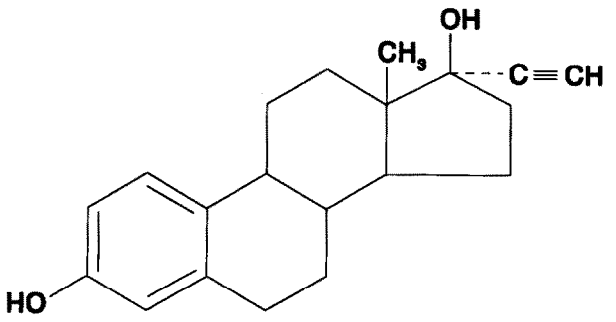

Figure 1
Chemical structure of ethinylestradiol.

* Author to whom correspondence should be addressed.

alter circulating levels of EE2 [15]. Thus sensitive and accurate assay methods are needed to monitor the low levels of EE2 present during therapeutic regimens.

Assay of EE2 by gas chromatography–mass spectroscopy [16] is reliable and sensitive, but entails specialized equipment and high expense that may be prohibitive for many applications. Liquid chromatography (LC) coupled with radioimmunoassay was shown by Lee *et al.* [17] to resolve EE2 from potential cross-reactants, although existing immunoassays are susceptible to concerns of sensitivity and interference or cross-reactivities of other matrix components.

This report describes an assay procedure for EE2 that combines sequential stages of solid-phase plasma extraction, LC, and a highly sensitive radioimmunoassay. This protocol addresses effectively the issues of sensitivity, precision, specificity, stability, and repeatability, for accurate quantification of low circulating levels of this prominent oestrogen analogue.

Materials and Methods

Materials

Ethinylestradiol (19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol), norethindrone (17-hydroxy-19-norpregn-4-en-20-yn-3-one), 17β-oestradiol, oestrone, equilin (3-hydroxyestra-1,2,5(10),7-tetraen-17-one) and other steroids were purchased from Sigma (St Louis, MO, USA). Tritiated steroids were purchased from Dupont-NEN (Boston, MS, USA). Ethinylestradiol-6-O-carboxymethyloxime-bovine serum albumin was obtained from Steraloids (Wilton, NH, USA). Pooled normal human plasma was purchased from Valley Biomedical (Winchester, VA, USA). $Na^{125}I$ was provided by Amersham (Arlington Heights, IL, USA), C-18 cartridges were purchased from J.T. Baker (Phillipsburg, NJ, USA), and LC solvents were obtained from Baxter (McGraw, IL, USA).

Plasma extraction

Procedures were conducted using normal human plasma, some of which received known quantities of analyte. Plasma samples were extracted using C-18 cartridges (3 ml/200 mg) on a Vac-Elute manifold. To monitor steroid recoveries, approximately 1 nCI of tritiated tracer (repurified using the LC system described below) was added to each sample (2 ml), which were then acidified with 4.0 ml of 50 mM HCl. Samples were applied to cartridges that had been preconditioned with 3.0 ml methanol and 2 × 3 ml of deionized H_2O. Sample vials were rinsed with 3 ml of 5 mM HCl which were applied to columns, then columns were flushed sequentially with 3 ml of 5 mM HCl followed by 2 × 3 ml of H_2O. Samples were eluted from columns with 1 ml methanol, extracts were evaporated to dryness on a Zymark TuboVap, and residues were resuspended in 200 μl methanol before LC.

Liquid chromatography

Liquid chromatography was conducted using the procedure of Lee *et al.* [17]. Using a WISP autosampler coupled to a Waters HPLC apparatus, samples were applied to Alltech LiChrosorb RP-8 column (10 μm, 250 × 4.6 mm i.d.) and eluted with H_2O–acetonitrile–methyl *t*-butyl ether (65:35:10, v/v/v) at a flow rate of 2 ml min^{-1}. Eluates were collected with a ISCO Foxy automatic fraction collector and either assessed for tracer content with an ICN Taurus liquid scintillation counter or evaporated to dryness on a Savant Speed Vac for radioimmunoassay.

Preparation of antiserum

A modification of the method of Vaitukaitis *et al.* [18] was used to immunize six white New Zealand rabbits with ethinyloestradiol-6-O-carboxymethyloxime:BSA.

The rabbits were sensitized with injection of 600 μl (6×10^{10} cells) killed *Bordetella pertussis* intramuscularly 4 days prior to being immunized at 30–40 sites intradermally with 500 μg of the steroid conjugate homogenized in complete Freunds adjuvant. Booster injections of 250 μg conjugate in incomplete adjuvant were administered every 3 weeks thereafter.

Trial bleeds were taken 10 days following each booster injection. Bleeding date 7 May 1991 from animal No. 608 was used in subsequent procedures.

Iodination

Ethinylestradiol was iodinated [19] directly for use as a radiotracer in radioimmunoassays. One mCi of $Na^{125}I$ was added to 2.5 μg EE2 in 50 μl 0.1 M borate (sodium tetraborate, pH 8.6) buffer. Iodination was initiated with 10 μl

of 17.6 mM chloramine T and quenched after 90 s with 10 μl of 21 mM sodium metabisulphite. Iodinated EE2 was isolated by LC, with elution using a solvent gradient of 20–80% acetonitrile–H_2O (v/v). Specific activity of ^{125}I-EE2 was approximately 1900 Ci mmol^{-1} as calculated by "self-displacement" assay [20].

Radioimmunoassay

Ethinylestradiol was quantified by double antibody radioimmunoassay. All radioimmunoassay components were added in buffer containing 8.0 g NaCl, 1.07 g Na_2HPO_4, 0.2 g KCl, 0.2 g KH_2PO_4, 0.1 g $MgCl_2$–$6H_2O$, 0.1 g $CaCl_2$–$2H_2O$, 0.06% gelatin, 0.04% bovine serum albumin, and 1.0 g NaN_3 per litre, pH 7.4. Samples taken from LC were resuspended in 1.0 ml assay buffer by incubation at 37°C for 30 min with repeated agitation. The tritium content of sample aliquots was quantified to correct for steroid recovery. Radioimmunoassay tubes contained 10,000 cpm ^{125}I-EE2, 1:800,000 final dilution of antiserum, and sample or standard, in a total volume of 500 μl. Standard curves were established in tubes containing doubling dilutions of EE2 from 16 to 0.125 pg/tube. Total binding (zero dose) and nonspecific binding were assessed in tubes in which buffer replaced sample and antiserum, respectively. Total radioactivity was monitored in tubes containing only assay tracer, and quality control samples were routinely established using charcoal-stripped plasma containing 0, 6, 18, or 54 pg ml^{-1} EE2. Standards were conducted in triplicate, and samples in duplicate. Assays were incubated for 40 h at 2–6°C, then 300 μl normal rabbit serum (1:175) and 300 μl of goat anti-rabbit antiserum (1:15) were added to assay tubes followed by a second incubation at 2–6°C for 2 h. Assay tubes (except total radioactivity tubes) then received 3.0 ml 7.5% polyethylene glycol and were centrifuged for 45 min at 2–6°C and at 2500*g*. Supernatants were discarded and radioactivity was quantified in pellets using an ICN Apex scintillation counter.

Radioactivity in radioimmunoassay samples and standards was converted to %B/Bo values (%B/Bo = (cpm in sample or standard − cpm nonspecific binding)/(cpm in zero dose standards − cpm nonspecific binding)). Standard curves were fitted to a four-parameter logistic data reduction program and samples were quantified using Sigmoid [21].

Assay validation procedures

The capacity of Sigmoid to fit standard curves to experimental data was evaluated by back-fitting observed standard values to calculated values [21]. The reproducibility of radioimmunoassay standards curves was monitored by comparison of the standard curves generated in six independent assays. Parameters were established for assay sensitivity (defined as the lowest plasma concentration that could be reproducibly measured with an inter-assay coefficient of variation ≤20%), accuracy (%accuracy = 100 (mass measured/mass added)), intra-assay precision (mean % coefficient of variation for six replicates each of three quality control pools, ranging from 6 to 54 pg ml^{-1}, run in one assay), inter-assay precision (defined as the mean % coefficient of variation for single replicates of the three quality control pools run in six assays), and recovery (measurement of the % recovery of added ^{3}H-EE2 and ^{3}H-norethindrone through the extraction and LC phases). Parallelism was assessed by simultaneous assay of selected samples at multiple dilutions.

Specificity of the radioimmunoassay was determined using samples containing graded concentrations of selected steroids. Percentage cross-reactivities were calculated as 100 (molar concentration of EE2 at ED_{50}/molar concentration of cross reactant at ED_{50}) where ED_{50} is the concentration that caused 50% reduction in specific binding.

Stability of EE2 in plasma matrix quality control samples was assessed after four freeze–thaw cycles. In each cycle, duplicate aliquots of plasma containing 0 (charcoal-stripped plasma), 6, 18 or 54 pg ml^{-1} EE2 were removed from storage at −20°C, allowed to thaw at ambient temperature for 2 h, then refrozen. Frozen–thawed samples were then assayed in parallel with aliquots that had not been subjected to the freeze–thaw cycles.

Stability of purified analyte in radioimmunoassay buffer solution was evaluated. Aliquots of sample extracts from LC were stored at 2–6°C for 3 weeks before radioimmunoassay.

Results

Recoveries of EE2 from the plasma extraction and chromatography procedures were determined. The mean recovery of ^{3}H-EE2 added to 42 samples that were then subjected to extraction and chromatography was 76.1 ±

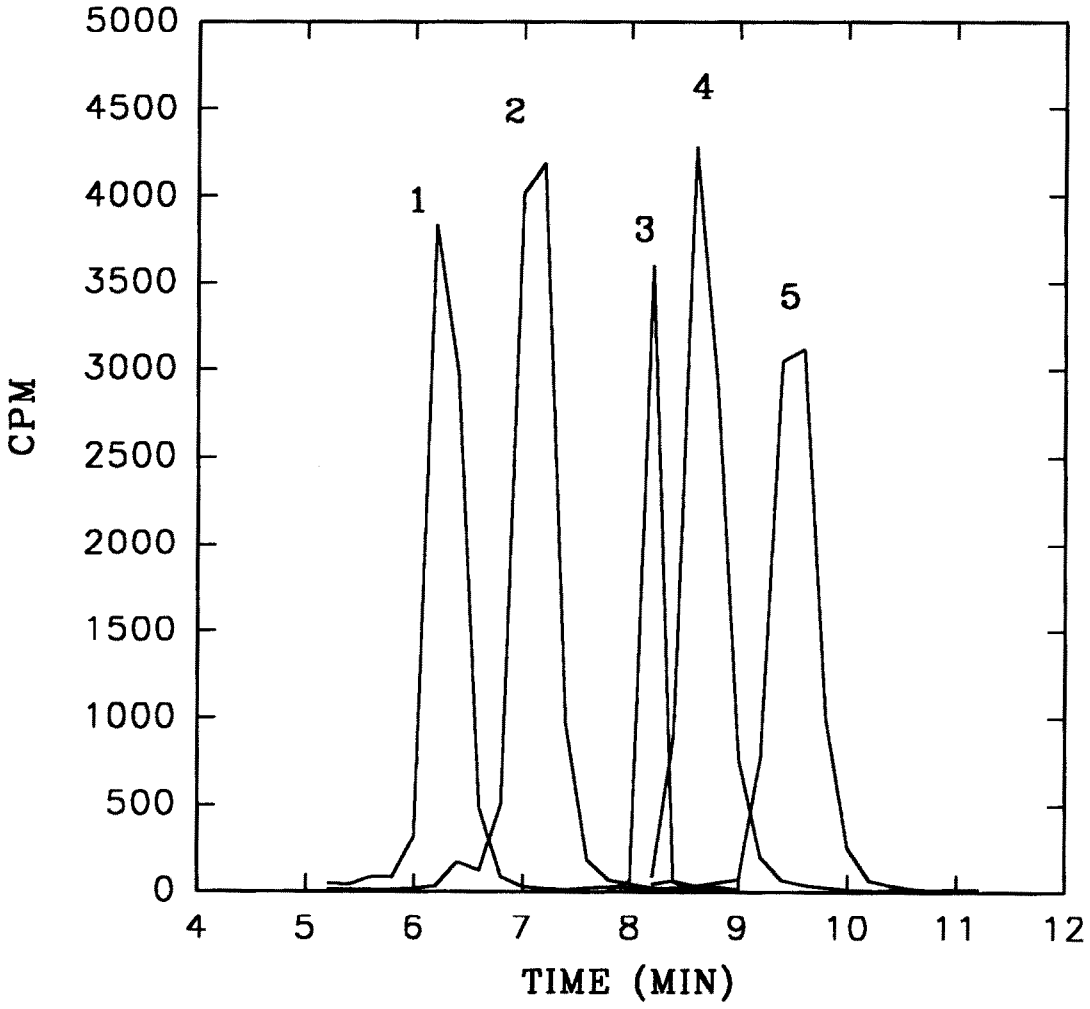

Figure 2
Composite chromatogram depicting separation of norethindrone (1), 17β-oestradiol (2), equilin (3), oestrone (4), and ethinylestradiol (5) in extracted plasma samples by liquid chromatography. Tritiated steroids were applied to an RP-8 column and eluted with water–acetonitrile–methyl *t*-butyl ether (65:35:10, v/v/v) and a flow rate of 2 ml min^{-1}.

Table 1
Standard curve backfit values

	Nominal concentrations (pg/tube)							
	0.125	0.250	0.500	1.00	2.00	4.00	8.00	16.0
Assay	Back-fit concentrations (pg/tube) $\bar{x}n = 3$							
VRP2	0.194	0.198	0.497	0.967	2.06	4.02	8.08	15.3
VRP3	0.183	0.221	0.530	0.912	2.10	4.02	7.96	15.9
VRP4	0.150	0.217	0.516	0.964	2.05	4.09	7.72	16.3
VRP5	0.165	0.202	0.524	0.968	2.01	4.10	8.10	14.7
VRP6	0.167	0.196	0.556	0.976	1.97	4.07	8.05	16.0
VRP8	0.155	0.234	0.472	1.01	2.04	4.04	7.85	16.1
Mean	0.169	0.211	0.516	0.966	2.04	4.06	7.96	15.7
SD	0.0167	0.0151	0.0288	0.0315	0.044	0.035	0.150	0.60
% CV	9.88	7.16	5.58	3.26	2.16	0.862	1.88	3.82
% Error	35.2	−15.6	3.20	−3.40	2.00	1.50	−0.500	−1.88
$\bar{x}$ %B/Bo	92.2	90.2	78.0	63.8	43.9	26.7	14.8	7.66
$\bar{x}$ pg ml^{-1}	1.10	1.38	3.36	6.49	13.3	26.5	51.9	102.0

*Concentration equivalent to a 2 ml sample corrected for average recovery of 0.767.

4.5% ($\bar{x}$ ± SD). Recovery of ^{3}H-norethindrone to the same 42 samples was 74.5 ± 4.5%, and recovery of ^{3}H-norethindrone to a separate set of 42 samples was 76.7 ± 3.6%. Since recoveries of EE2 and norethindrone were not different, recovery of norethindrone was considered to be a reliable estimate of EE2 recovery and was used in subsequent assay development procedures.

The LC procedure resolved steroids that were considered to be candidates for subsequent assay cross-reactivity. A representative chromatogram is depicted in Fig. 2.

Mean values for six radioimmunoassay standard curves are depicted in Fig. 3. When observed analyte concentrations were back-fit to the theoretical standard curve using Sigmoid, all concentrations above 0.25 pg differed from theoretical with coefficients of variation <6% (Table 1).

Sensitivity of the radioimmunoassay, defined as the lowest amount of EE2 that could be quantified with coefficient of variation ≤20%, was approximately 0.4 pg/tube, which corresponded before the extraction and chromatography procedures to approximately 2 pg ml^{-1} plasma (Fig. 3).

In six separate assays, samples containing 2, 3, 4, 6, 18 and 54 pg ml^{-1} EE2 produced mean values of 2.2, 3.0, 3.8, 6.0, 18.6 and 55.4 pg ml^{-1}, respectively. Defining accuracy as 100 times the mass measured divided by the mass added, mean assay accuracy within this range of EE2 concentrations was 101.5%. Intra-assay coefficient of variation was 3.6% and inter-assay coefficient of variation was 8.9%.

Selected samples were serially diluted before radioimmunoassay. Dilution profiles were parallel, and analyte content in dilutions were proportional to dilution magnitude (Fig. 4).

Samples that were subjected to four freeze–thaw cycles were assayed (Fig. 5). Up to four cycles of freeze–thaw did not decrease measured amounts of EE2. Samples that were stored at 2–6°C for 2 weeks yielded EE2 values that were not different from levels without storage (Fig. 5).

Cross-reactivity of selected steroids in the radioimmunoassay is depicted in Table 2. Marked cross-reactivity by equilin, and moderate cross-reactivities by norethindrone, oestradiol, and oestrone, were detected.

Discussion

Sensitive and accurate assays for EE2 are

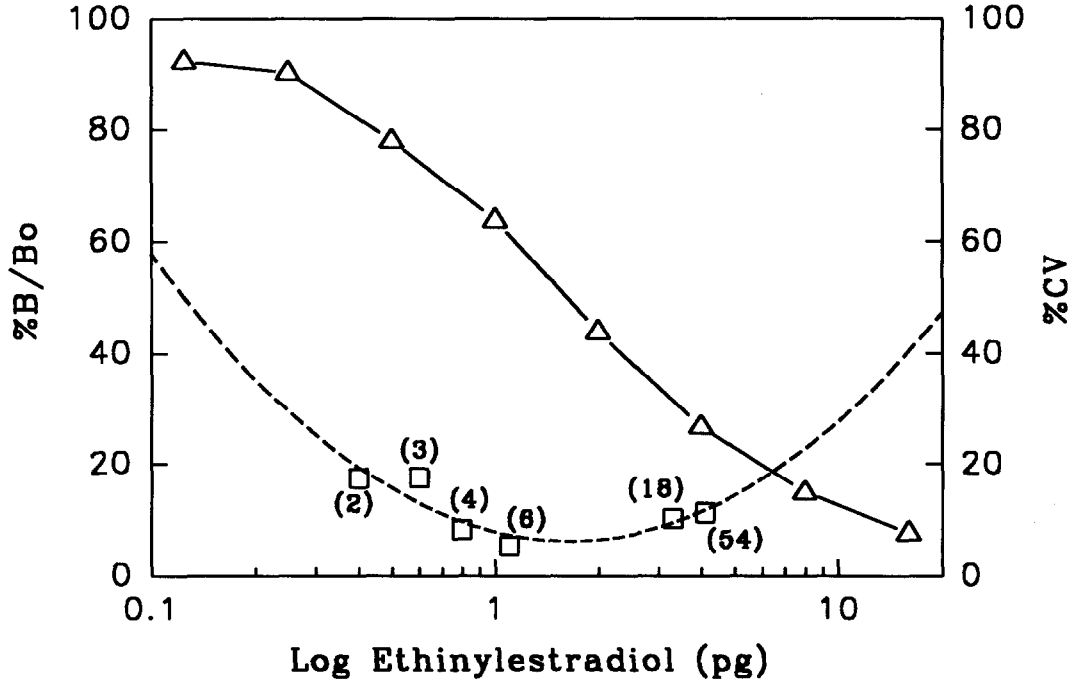

Figure 3
Ethinylestradiol radioimmunoassay standard curve and precision profile. Triangles depict mean binding of standards as a function of ethinylestradiol mass in six separate assays. Squares depict coefficients of variation for six different assays of sample pools. Numbers in parentheses depict the ethinylestradiol concentrations (in pg ml^{-1}) in the sample pools prior to the procedures of extraction, chromatography, recovery estimates and radioimmunoassay. The dashed line depicts a second-order fit of the %CV data.

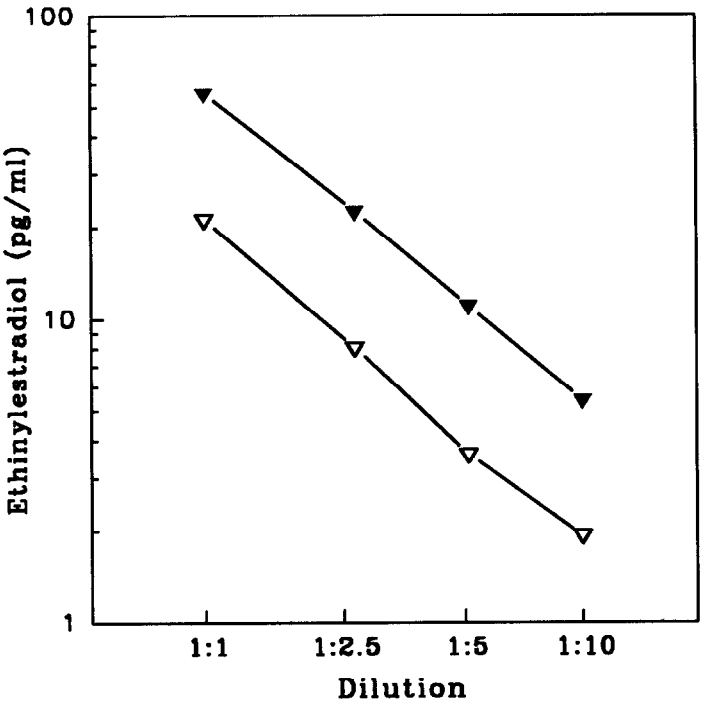

Figure 4
Parallelism of sample dilutions. Samples containing 18 or 54 pg ml^{-1} ethinylestradiol were serially diluted then assessed in radioimmunoassay.

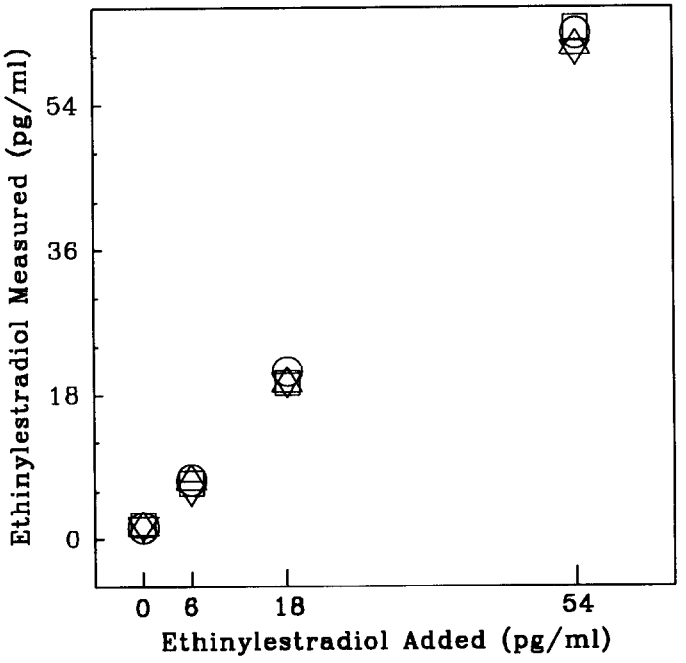

Figure 5
Stability of ethinylestradiol to freeze–thaw cycles and storage in buffer. Samples were extracted, chromatographed, and quantified by radioimmunoassay (inverted triangles). Quantification procedures were conducted on additional samples (squares and triangles) that received four freeze–thaw cycles before extraction, or were stored in assay buffer for 2 weeks after chromatography (circles). Measured ethinylestradiol in all samples containing O-ethinylestradiol (charcoal-stripped plasma) was consistently below the minimum quantifiable assay level.

Table 2
Cross-reactivity of selected steroids

Steroid	% Cross-reaction at ED_{50}
Ethinylestradiol	100
Equilin	28.2
Norethindrone	8.4
17β-oestradiol	3.1
Oestrone	<0.5
Testosterone	0.2
Oestriol	0.02
Oestrone sulphate	0.003
Cortisol	0.003
Progesterone	0.002
Dehydroepiandrosterone sulphate	0.002
Androstenedione	<0.002
Dehydroepiandrosterone	<0.002

needed. To serve as an oral contraceptive with minimalization of untoward side effects, daily doses of as little as 20 μg EE2 are typical [22]. Although efficiently absorbed from the gastrointestinal tract in humans, EE2 is subject to extensive first pass metabolism consisting chiefly of conjugation with sulphate in the gut wall [23]. Conjugated EE2 is poorly absorbed from the gut but enterohepatic recirculation can render additional EE2 in unconjugated form for absorption [4]. EE2 is cleared from circulation with a half-life of approximately 6 h [3, 24]. In typical contraceptive use, plasma levels of EE2 are in the range of 10–100 pg ml^{-1} [4, 25], although circulating EE2 levels exhibit high interindividual and intraindividual variability [26]. Thus, EE2 administered in oral contraceptives circulates in a concentration range that efficiently blocks ovulation but challenges conventional detection and quantification methodologies.

Prior methods to quantify circulating EE2 and other contraceptive steroids using liquid chromatography offered excellent resolution but insufficient sensitivity [27, 28], necessitating doubling of normal doses for pharmacokinetic studies. Most existing radioimmunoassays for EE2 are compromised by problems of crossreactivity by other, structurally similar steroids [24]. Coupling of LC with a highly sensitive radioimmunoassay system represented a route to alleviate the twin problems of sensitivity and cross-reactivity.

The radioimmunoassay described herein had high sensitivity for EE2, adequate to quantify low concentrations of EE2 that circulate during typical therapeutic regimens. The high sensitivity of this radioimmunoassay was attributable to several features. The EE2 antiserum produced by Hazleton had high affinity for EE2, as evidenced by optimal binding parameters when used at 1:800,000 final dilution. The iodinated EE2 used as tracer for radioimmunoassay had a specific activity (1900 Ci $mmol^{-1}$) approximately 30-fold higher than commercially available ^{3}H-EE2.

Assay sensitivity was maximized also by the use of ^{3}H-norethindrone to monitor EE2 recovery from plasma samples. Sample spiking with ^{3}H-norethindrone provided reliable estimates of EE2 recovery. In 42 samples spiked with both ^{3}H-EE2 and norethindrone evaluated in six assays, recovery of EE2 and norethindrone through the extraction and LC steps were not different (76.1 ± 4.5% vs 75.5 ± 4.5%). Thus the mass effect of added EE2 on assay sensitivity was eliminated by using norethindrone as recovery tracer.

Plasma extraction was necessary before the LC step to separate EE2 from plasma proteins. Less than 2% of administererd EE2 is found free in circulation; most is bound to albumin [29]. Following plasma extraction, LC was necessary to separate EE2 from other steroids. The antiserum used in this study, like most other EE2 antisera, exhibited partial cross-reactivity (Table 1) with several other oestrogens and norethindrone. However, LC clearly resolved EE2 from other steroids that were potential candidates for assay interference (Fig. 2).

The assay procedure for EE2 was validated for parameters of accuracy, reproducibility, sensitivity, recovery, stability and cross-reactivity. By all measures, the assay is adequate to determine with high confidence therapeutic levels of EE2 in circulation.

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References

[1] I.H. Thorneycroft, *Adv. Contracept.* **8**(Suppl. 1) 5–12 (1992).
[2] W. Kuhnz, T. Louton, D.J. Back and K. Michaelis, *Arzneimittelforschung* **43**, 16–21 (1993).
[3] J.R. Pasqualini, R. Castellet, M.C. Portois, J.L. Hill and F.A. Kincl, *J. Reprod. Fertil.* **49**, 189–193 (1977).
[4] M.L.'E. Orme and D.J. Back, *Am. J. Obstet. Gynecol.* **163**, 2146–2152 (1990).

[5] J.W. Delahunt, *Drugs* **45**, 223–231 (1993).
[6] P. Henriksson, B. Angelin and L. Berglund, *J. Clin. Invest.* **89**, 1166–1171 (1992).
[7] G.B. Cutler, Jr and J.L. Ross, *Acta Paediatr. Jpn.* **34**, 195–205 (1992).
[8] N. Stahnke and H. Koehn, *Horm. Res.* **33**(Suppl. 4), 38–44 (1990).
[9] H. Svan, E.M. Ritzén, K. Hall and L. Johansson, *Acta Paediatr. Scand.* **80**, 328–332 (1991).
[10] F. Anderson, *Int. J. Fertil.* **38**(Suppl. 1), 53–64 (1993).
[11] L. Falsetti and E. Galbignani, *Contraception* **42**, 611–619 (1990).
[12] M. Nabrink, L. Birgersson, A.S. Colling-Saltin and T. Solum, *Contraception* **42**, 275–283 (1990).
[13] A.M. Webb, *Adv. Contracept.* **7**, 271–279 (1991).
[14] P.R. Kenya, *East Afr. Med. J.* **67**, 146–153 (1990).
[15] D.J. Back and M.L'E. Orme, *Clin. Pharmacokinetic.* **18**, 472–484 (1990).
[16] E. Daeseleire, A. De Guesquière and C. Van Peteghem, *J. Chromat.* **564**, 469–475 (1991).
[17] G.J.-L. Lee, M.-H. Oyang, J. Bautista and S. Kushinsky, *J. Liquid Chromatogr.* **10**, 2305–2318 (1987).
[18] J. Vaitukaitis, J.B. Robbins, E. Nieschlag and G.T. Ross, *J. Clin. Endocrinol.* **33**, 988–991 (1971).
[19] F.C. Greenwood, W.M. Hunter and J.S. Glover, *Biochem. J.* **89**, 114–123 (1963).
[20] B.J. Morris, *Clin. Chim. Acta* **73**, 213–216 (1976).
[21] D. Rodbard and D. Hutt, in *RIA and Related Procedures in Medicine*, pp. 165–192. International Atomic Energy Agency, Vienna (1974).
[22] G.B. Melis, F. Fruzzetti, I. Nicoletti, C. Ricci, P. Lammers, W.J. Atsma and P. Fioretti, *Contraception* **43**, 23–31 (1991).
[23] M. Orme and D.J. Back, *Adv. Contracept.* **7**, 325–331 (1991).
[24] G.M. Shenfield and J.M. Griffin, *Clin. Pharmacokinet.* **20**, 15–37 (1991).
[25] U. Täuber, W. Kuhnz and M. Hümpel, *Am. J. Obstet. Gynecol.* **163**, 1414–1420 (1990).
[26] J.W. Goldzieher and S.A. Brody, *Am. J. Obstet. Gynecol.* **163**, 2114–2119 (1990).
[27] G. Carignan, B.A. Lodge and W. Skakum, *J. Chromat.* **315**, 470–477 (1984).
[28] A.M. Bond, I.D. Heritage and M.H. Briggs, *J. Chromat.* **315**, 313–320 (1984).
[29] W. Kuhnz, M. Pfeffer and G. Al-Yacoub, *J. Steroid Biochem.* **35**, 313–318 (1990).

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