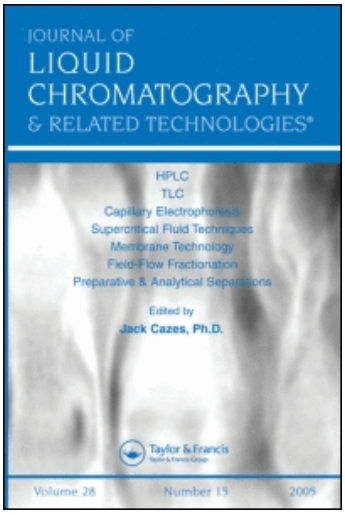

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Determination of Ethinylestradiol and Norethindrone in a Single Specimen of Plasma by Automated High-Performance Liquid Chromatography and Subsequent Radioimmunoassay

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DETERMINATION OF ETHINYLESTRADIOL AND NORETHINDRONE IN A SINGLE SPECIMEN OF PLASMA BY AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND SUBSEQUENT RADIOIMMUNOASSAY

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ABSTRACT

A method is described for the specific determination of ethinylestradiol and norethindrone in the same specimen of plasma at concentrations of 20 and 50 pg/ml, respectively. The method is based on fully automated high-performance liquid chromatography for separation of the analytes form one another and from coextracted extraneous components and on radioimmunoassay using antisera of high specificity for the quantification. Validation of the method included demonstration of low procedural blanks, recoverability of added hormones and low intra-assay and inter-assay variability.

INTRODUCTION

Several radioimmunological procedures have been reported for the determination of ethinylestradiol (EE_2) and/or norethindrone (NET) in plasma (1-5). Because the antisera employed in the assays are not completely specific, it generally is necessary to include a fractionation procedure to avoid interference from the other analyte (if both are present in the same specimen), from metabolites of the analyte(s) and from crossreactive endogenous steroids (4-7). The latter are of particular concern for assays of EE_2 in plasma from premenopausal women. In the method described here, fully automated high-performance liquid chromatography (HPLC) is used to separate EE_2 and NET from one another and from potentially interfering endogenous steroids prior to radioimmunoassay (RIA).

MATERIALS AND METHODS

Materials and Reagents

Ethinylestradiol and norethindrone were products of Syntex Incorporated (Palo Alto, Calif.). 6,7-^{3}H-Ethinylestradiol (57.3 Ci/mmole) and 6,7-^{3}H- norethindrone (55.0 Ci/mmole) were purchased from New England Nuclear (Boston, Mass.) and purified by HPLC prior to use. Stock solutions of non-radiolabeled steroids were

prepared in methanol at a concentration of 100 µg/ml. From this stock solution, different concentrations of solutions for RIA standards (ranging from 4000 pg to 1.56 pg/0.2 ml) were made by serial dilution with 0.1 M phosphate buffer (pH 7.0) containing 0.1% bovine gamma-globulin.

The following reduced metabolites of norethindrone: 17α-ethinyl-17β-hydroxy-5β-estran-3-one; 17α-ethinyl-17β-hydroxy-5α-estran-3-one; 17α-ethinyl-5α-estrane- 3α, 17β-diol; 17α-ethinyl-5α-estrane-3β, 17β-diol; 17α-ethinyl-5β-estrane-3β, 17β-diol; 11α, 17β-dihydroxy-17α-ethinyl-4-estren-3-one; 16β, 17β-dihydroxy-17α-ethinyl-4-estren-3-one and 16α, 17β-dihydroxy-17α-ethinyl-4-estren-3-one were provided by Dr. F. Z. Stanczyk (Oregon Primate Research Center, Beaverton, Ore., U.S.A.).
Two reduced metabolites of norethindrone, 17β-hydroxy-17α-ethinyl-5α-estran-3-one and 17α-ethinyl-5α-estrane-3α, 17β-diol were provided by Dr. H. Watanabe (Health and Welfare Canada, Ottawa, Ontario, Canada).

Dextran-coated charcoal solution was prepared by mixing 500 mg of charcoal, 50 mg of Dextran T 70 and 100 ml of

0.1 M phosphate buffer solution (pH 7.0). The 0.1 M phosphate buffer contained 0.9% sodium chloride and 0.1% sodium azide. The antiserum for RIA of EE_2, prepared by immunizing rabbits against EE_2-7-(3-thiopropionic acid)-bovine serum albumin, was provided by Dr. C. E. Cook (Research Triangle Institute, Research Triangle Park, N.C.). The norethindrone antiserum was provided by Dr. L.R. Beck (University of Alabama, Birmingham, Ala.) or purchased from Cambridge Medical Diagnostics (Billerica, Mass.).

HPLC Equipment

The HPLC system employed in this work included a Model 6000A pump, a Model 45 pump, a Model 710B autosampler, a Model 720 system controller and a Model 730 data module (Waters Assoc., Milford, Mass.). A Model 328 fraction collector from ISCO (Lincoln, Neb.) was used in conjunction with HPLC equipment. A three-way slider valve equipped with a solenoid operated pneumatic actuator (Altex, Berkeley, Calif.) mounted on the fraction collector was used for collecting the desired fractions and transferring the other fractions to a waste bottle. The column (25 cm x 4.6 mm I.D., Alltech Associates, Deerfield, Ill. or Chromanetics Corp., Kensington, Md.) was prepacked with 10-µm LiChrosorb RP-8 and protected by a guard column system containing a

3 cm cartridge in a stainless steel holder (Brownlee Labs, Santa Clara, Calif.).

Solvent Extraction and Preliminary Purification of Plasma

To a test tube were added 1 ml of human female plasma and 0.1 ml each of solutions containing 600 dpm of ^{3}H-EE_2 or 500 dpm of ^{3}H-NET in gamma-globulin phosphate buffer. In tests of the recoverability of added steroid, each of the designated quantities of unlabeled steroid in 0.2 ml of gamma-globulin phosphate buffer was added likewise. To the resulting solutions was added 5 ml of mixture of hexane and ethyl acetate (2:1, by volume). The extraction was carried out on a rotating extractor for 10 minutes. After centrifugation at 1500 rpm for 10 minutes, the upper phase was pipetted into another tube and evaporated to dryness under a stream of nitrogen in a 50°C water bath. The wall of the tube was rinsed twice with 0.6 ml of acetone, with evaporation to dryness after each rinse. The residue was dissolved in a small volume (240 µl) of 50% aqueous acetonitrile and the sample was passed through a 0.2 µm ACRO LC-13 filter (Gelman, Ann Arbor, Mich.) before application to the HPLC system.

Automated HPLC Separation

An automated HPLC system with two pumps was used in this study. The first pump delivered the mobile phase (water-

acetonitrile-methyl t-butyl ether; 65:35:10, V/V) that was used for separation of NET and EE_2. The second pump was used to deliver the solvent with stronger eluting characteristics (Table 1) to clean the column between each application of sample. This precaution helps to maintain the efficiency of the column, provides uniform retention times for the analytes and prevents the carryover of impurities from the preceding sample. Before each run, the exact collection time for each steroid was established by applying a mixture containing 5000 dpm of each tritiated steroid and collecting the effluent in 0.2-min fractions which were assayed for ^3H. The collection volumes were approximately 2.2 ml for NET and 3.0 ml for EE_2.

TABLE 1

Solvent Program for Automated HPLC

RUN TIME (min)	FLOW RATE (ml/min)	% Solvent A[a]	% Solvent B[b]
Initial	2.00	100	0
12.5	2.00	0	100
15.5	2.00	100	0

[a]Water-acetonitrile-methyl t-butyl ether (65:35:10 V/V)

[b]Acetonitrile

Radioimmunoassay

The effluent from the HPLC column corresponding to the EE_2 and NET peaks were evaporated to dryness separately at 50°C under a stream of nitrogen. After rinsing down with 1 ml of ethyl acetate and evaporation of the solvent to dryness, the residue was redissolved in 0.6 to 1.0 ml of gamma-globulin phosphate buffer by incubation in a water bath at 50°C for 30 minutes. The quantity of buffer was selected in accordance with the expected concentration of steroid. This expedient generally provided results that were within the useful range of the standard curves. One fifth of the solution was used for monitoring of procedural losses and 0.2 ml was used for the RIA. Further dilutions were done, if necessary.

For the RIA, diluted antiserum (1:10,000 for NET and 1:15,000 for EE_2) was added to an equal volume of the corresponding ^3H-labeled standard in protein-phosphate buffer (30,000 dpm/ml)and 0.2 ml of the resulting solution (3,000 dpm) was mixed with either 0.2 ml of sample or standard solution. Aliquots (0.2 ml) of standards in protein-phosphate buffer containing 0, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200 or 400 pg of NET or EE_2 were assayed in triplicate to establish a standard curve. Aliquots (0.2 ml) of samples were assayed in duplicate. In addition, a large excess of

non-radioactive steroid (4 ng of either NET or EE_2) was processed similarly in triplicate and the results were used for determination of the non-specifically bound (NSB) fraction. The solutions containing anti-serum, ^{3}H-steroid and unlabeled steroid (sample or standard) were incubated at 37°C for 30 minutes and then in an ice bath for 2 hours. While keeping the assay tubes in an ice bath, 0.4 ml of magnetically stirred, cold suspension of dextran-coated charcoal was added to each tube and the mixtures were incubated for an additional 1 hour. After centrifugation at 0°C for 10 minutes, the supernatant was poured into a liquid scintillation vial containing 10 ml of Biofluor. Radio-activity was determined in a liquid scintillation spectrometer with a paper-punch tape output (10-minute count for RIA tubes; 20-minute count for recoveries). Results were calculated automatically by means of a non-linear logistic program with appropriate corrections for procedural recoveries and aliquots used.

RESULTS

In Figure 1 is shown the HPLC separation of EE_2, NET, estrone, estradiol, testosterone and progesterone within 12.5 minutes. Use of methyl t-butyl ether as a solvent modifier in reversed-phase HPLC provided an enhanced separation comparable to that which we reported earlier

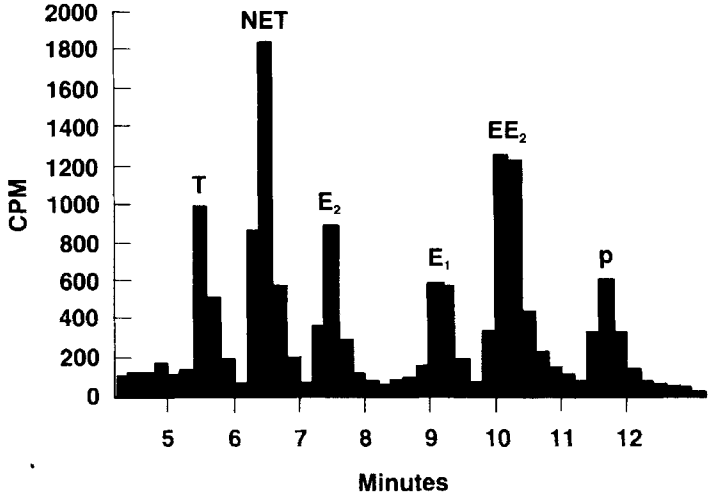

Figure 1. HPLC separation of ethinylestradiol (EE_2), norethindrone (NET), testosterone (T), estradiol (E_2), estrone (E_1) and progesterone (P); data based on determination of ^3H in 0.2-min fractions. column: Alltech LiChrosorb RP-8, 10 µm, 25 cm x 4.6 mm I.D.; solvent system: water-acetonitrile-methyl t-butyl ether (65:35:10 v/v); flow-rate: 2 ml/min; pressure: 2200 p.s.i.

for diethyl ether (8), with much less likelihood of oxidative degradation by peroxides. Some metabolites of NET, such as 17α-ethinyl-17β-hydroxy-5β-estran-3-one, 17α-ethinyl-17β-hydroxy-5α-estran-3-one and 11α, 17β-dihydroxy-17α-ethinyl-4-estren-3-one were reported to cross-react significantly with the NET antiserum (9,10). Based on the retention times of the eight reduced NET metabolites as listed in the Materials section, all of these metabolites were separated

completely from NET during the HPLC step. The average procedural recovery (extraction and HPLC) based on monitoring of losses of ^{3}H- steroids was determined (n = 30) to be 63.0 $\pm$ 3.67% (S.D.) and 73.2 $\pm$ 4.33% (S.D.) for EE_2 and NET, respectively. Procedural blanks were negligible for both EE_2 and NET. Standard curves for the RIA of EE_2 and NET indicated that a statistically significant reduction of antibody-bound ^{3}H was found routinely for 3.13 pg of both EE_2 and NET but only intermittently for 1.56 pg of the analytes. Accuracy of the procedures was assessed by determination in triplicate of the recovery of four different quantities of EE_2 and of three different quantities of NET added to 1 ml of plasma. The results of two such studies done on different days are summarized for EE_2 and NET in Tables 2 and 3, respectively. Plots of the average of the measured values versus the quantities added, yielded regression lines described by the following equations, $y = 0.93 x + 4.91$ ($r = 0.999$) and $y = 0.96 x - 0.15$ ($r = 1.000$) for EE_2 and NET, respectively. Based on the results of these experiments, the sensitivity of the method is 20 pg/ml for EE_2 and 50 pg/ml for NET.

An average intra-assay variability of 6.73% was calculated for EE_2 from the results of the two spiking

TABLE 2

Recovery of Ethinylestradiol Added to Control Plasma

Run No.	Quantity Added (pg/ml)	Quantity Measured (Mean, pg/ml)	Standard Deviation (pg/ml)	Coefficient of Variation (%)	Percent Recovered (%)
1.	20	19.1	1.16	6.07	96
	50	52.2	3.22	6.17	104
	100	101	9.63	9.54	101
	200	176	8.63	4.90	88
2.	20	22.0	2.77	12.6	110
	50	52.2	3.70	7.09	104
	100	103	2.35	2.28	103
	200	202	10.5	5.20	101

TABLE 3

Recovery of Norethindrone Added to Control Plasma

Run No.	Quantity Added (pg/ml)	Quantity Measured (Mean, pg/ml)	Standard Deviation (pg/ml)	Coefficient of Variation (%)	Percent Recovered (%)
1.	50	49.7	3.98	8.01	99
	100	96.8	4.90	5.06	97
	200	185	14.1	7.62	93
2.	50	46.5	3.89	8.37	93
	100	94.2	6.80	7.22	94
	200	198	9.04	4.57	99

experiments summarized in Table 2 and of 6.81% for NET from the results summarized in Table 3. Analysis of the results of 15 sequential experiments in which control plasma was spiked in duplicate with 100 pg of EE_2 and with 100 or 200 pg of NET yielded average inter-assay variabilities of 12.6 and 13.5% for EE_2 and NET, respectively.

DISCUSSION

The method described here was developed for use in a bioequivalence study of oral contraceptive formulations containing EE_2 and NET. The content of NET in the formulation was approximately 30 times that of EE_2. Consequently, the separation of NET from EE_2 was necessary in order to avoid an overestimation of EE_2 through cross-reactivity of NET with the EE_2 antiserum. In addition, since the study was to be conducted in premenopausal women, it also was necessary to separate the endogenous estrogens from EE_2 prior to RIA. Inclusion of an HPLC separation in the procedure eliminates both the possibility of significant interference by NET and by potentially cross reactive endogenous steroids in plasma that vary considerably during the normal menstrual cycle. Although the cross reactivities of the EE_2 antiserum with NET or the major endogenous steroids are of the order of only 1% or

less, the quantification of EE_2 would not have been attainable without the HPLC separation. This method has recently been applied successfully to specimens of plasma obtained at various times after oral administration of tablets containing NET and EE_2 to normal premenopausal women (11). Use of fully automated HPLC for this separation is much more convenient than manual procedures. With this technique, 36 samples can be extracted conveniently each day and the extracts then loaded into an HPLC autosampler for overnight separation in preparation for RIA the following day. Thus, although the determinations involve both HPLC and RIA, the overall procedure is quite suitable for routine use in studies of bioavailability or bioequivalence.

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