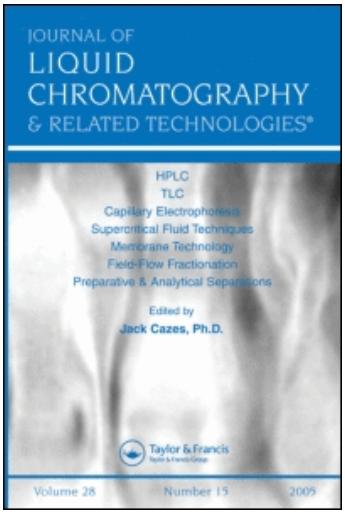

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Determination of Norgestimate in Serum by Automated High-Performance Liquid Chromatography and Subsequent Radioimmunoassay

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DETERMINATION OF NORGESTIMATE IN SERUM BY AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND SUBSEQUENT RADIOIMMUNOASSAY

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ABSTRACT

A method is described for the specific determination of norgestimate in human serum at concentrations of 25 to 1000 pg/mL. The method is based on automated high-performance liquid chromatography and on subsequent radioimmunoassay. Validation of the method included demonstration of low procedural blanks, recoverably of added standard and low intra- and inter-assay variability.

INTRODUCTION

Norgestimate is a progestin synthesized by The R. W. Johnson Pharmaceutical Research Institute at Ortho Pharmaceutical Corporation.

It has been extensively investigated clinically as an oral contraceptive. To measure serum norgestimate concentrations following oral administration, a radioimmunoassay was developed. The measurement of norgestimate in biological fluids by radioimmunoassay alone presented a problem because the antisera employed in the existing assay was not completely specific. To overcome the uncertainties of immunological cross-reactivity and to provide assay specificity, an adjunct HPLC procedure was developed. This communication describes an automated high-performance liquid chromatography/radioimmunoassay (HPLC/RIA) method for the separation and quantitation of norgestimate in human serum.

MATERIALS AND METHODS

Materials and Reagents

Norgestimate(18,19-dinor-17-pregn-4-en-20-yn-3-one,17-(acetyloxy)-13-ethyl,oxine(17α(+)-) was synthesized at The R. W. Johnson Pharmaceutical Research Institute at Ortho Pharmaceutical Corporation (Raritan, NJ). 6,7-^{3}H-Norgestimate (44 Ci/mole) was purchased from Roussel-UCLAF (France) and purified by HPLC prior to use. The following metabolites of norgestimate: 3-keto-norgestimate (18,19-dinor-17-pregn-4-en-20-yn-3-one,17-(acetyloxy)-13-ethyl-(17α)-(+)-),17-deacetyl norgestimate (18,19-dinor-17-pregn-4-en-20-yn-3-one,17-hydroxy-13-ethyl,oxime), and norgestrel (18-19-dinor-17-pregn-4-en-20-yn-3-one,17-hydroxy-13-ethyl,(17α)-(+)-) were synthesized at The R. W. Johnson Pharmaceutical Research Institute at Ortho Pharmaceutical Corporation.

Stock solutions of non-radiolabelled norgestimate were prepared in

ethanol at a concentration of 2 mg/mL. From this stock solution, different concentrations of solutions for RIA standards (ranging from 20 to 1000 μg/mL) were obtained by serial dilution with 0.1 M phosphate buffer (pH 7.4) containing 0.1% bovine gamma-globulin and 0.01% Tween 80.

Dextran-coated charcoal tablets were obtained from Reflex Industries, Inc. (San Diego, CA).

The antiserum for the RIA for norgestimate, prepared by immunizing rabbits against the thyroglobulin conjugate of norgestrel-17-acetate-6-hemisuccinate, was provided by Dr. Delwood Collins at Emory University, Atlanta, Georgia.

HPLC-grade methanol, methyl-t-butyl ether, and ultrapure water were obtained from Burdick and Jackson Laboratories (Muskegon, Mich).

HPLC Equipment

The HPLC equipment employed in this work included a Model L-6200 pump, a Model L-4000 variable wavelength UV detector (Hitachi/EM Science, Cherry Hill, NJ), a Model ISS-100 autosampler (Perkin Elmer, Norwalk, Conn), and a 3357 Lab Automated System (Hewlett Packard, Piscataway, NJ). A Model 201/202 fraction collector fitted with a 3-way slider valve (Gilson, Middleton, WI) was used in conjunction with the HPLC equipment. The column (30 cm x 3.6 mm i.d.; Waters Millipore, Milford, Mass) was prepacked with 10 micron μ-Bondapak C_{18} and protected by a guard column system containing a 1.8 cm cartridge (Brownlee Labs, Santa Clara, CA).

Solvent Extraction and Purification of Serum

In tests of the recoverability of added norgestimate, 10 μL of a methanolic solution containing 50,000 dpm of ^{3}H-norgestimate was added to 1.0 mL portions of blank human female serum. To the resulting solutions was added 3 mL of methyl t-butyl-ether. The extraction was carried out by vortexing for 1 minute and centrifuging at 1500 cpm for 5 minutes. The organic (upper) phase was pipetted into another tube and evaporated to dryness under a stream of nitrogen at room temperature. The residue was then dissolved in a small volume (100 μL) of methanol before application to the HPLC system.

Automated HPLC Separation

In this study, an isocratic system incorporating methanol-water (80:20, v:v) at a flow rate of 1 mL/min was used. Before and during each daily run, the exact collection time for norgestimate was established by applying a mixture containing 20 ng of each of the unlabelled metabolite standards and observing the resulting retention times by UV. Under the conditions of the method, the approximate retention times of the standards were:

norgestimate (both syn and anti-isomers)	-	9.3-10 minutes
3-keto-norgestimate	-	7.7 minutes
deacetylated norgestimate	-	6.4 minutes
norgestrel	-	5.6 minutes

The collection volume for norgestimate was 2.0 mL obtained approximately 9-11 minutes after injection.

The effluent from the HPLC column corresponding to the norgestimate peak was evaporated to dryness at 40 °C under nitrogen. After evaporation, the residue was dissolved in 0.8 mL of gamma-globulin phosphate buffer. The quantity of buffer was selected in accordance with the expected concentration of steroid. This expedient provided results that were within the useful range of the standard curves.

For the RIA, diluted antiserum (1:3500) was added to an equal volume of the corresponding ^{3}H-labeled standard in protein-phosphate buffer and 0.2 mL of the resulting solution (4,000 cpm) was mixed with either sample or standard solution. Aliquots (0.05 mL) of standards in protein-phosphate buffer containing 0, 20, 40, 80, 100, 200, 400, 800, or 1000 pg of norgestimate were assayed in triplicate to establish a standard curve. Samples were assayed singly. In addition, a set of test tubes containing only buffer and ^{3}H-norgestimate was processed similarly in triplicate and the results were used to determine the nonspecifically bound (NSB) fraction. The solutions containing antiserum, ^{3}H-norgestimate or unlabelled norgestimate (sample or standard) were incubated at room temperature for 18 hours. A 40 mg dextran-coated charcoal tablet was then added to each tube and the mixtures were incubated for an additional 1 hour after centrifugation at 4 °C for 1 hour. The supernatant was poured into a liquid scintillation vial containing 15 mL of picofluor-15. Radioactivity was determined in a liquid scintillation spectrometer equipped with both hard copy (paper) and computer disc outputs (10 minute counts). Results were calculated automatically by means of a logit-log RIA data reduction program with appropriate corrections for procedural recoveries and aliquots used.

RESULTS

Figure 1 shows the separation of norgestimate (syn and anti-isomers) and three of its known metabolites. Based on the observed retention times, all of these metabolites were separated completely from norgestimate during the HPLC step. The average overall procedural recovery (extraction and HPLC fractionation) based on monitoring of losses of ^{3}H-norgestimate was determined to be 52 ± 5.2% (N=20).

Procedural blanks for the overall procedure were generally less than 15 pg/mL of serum.

Accuracy of the procedures was assessed by determining in triplicate, the recoveries of at least four different quantities of norgestimate added to 1 mL of serum. The results of such studies done on different days are summarized in Table 1. Plots of the averages of the measured values versus the quantities added yielded regression lines with r^2 values of 0.9981, 0.9967, and 0.9998, respectively. Based on the results of these experiments, the sensitively of the method for norgestimate is 25 pg/mL. Intra-assay variabilities of generally less than 15% were observed from the results of spiking experiments summarized in Table 1. Analysis of the results of these experiments in which blank serum was spiked in triplicate with amounts of norgestimate ranging from 50 to 500 pg/mL yielded inter-assay variabilities of generally less than 15%.

In a separate experiment, blank serum was spiked with 3-keto-norgestimate and norgestimate in the concentration ratios of 1/5, 1/1, and 5/1. The results of this experiment, as shown in Table 2, were quite similar to those in which the serum samples were spiked with norgestimate alone (Table 1), thus attesting to the specificity induced by the HPLC separation.

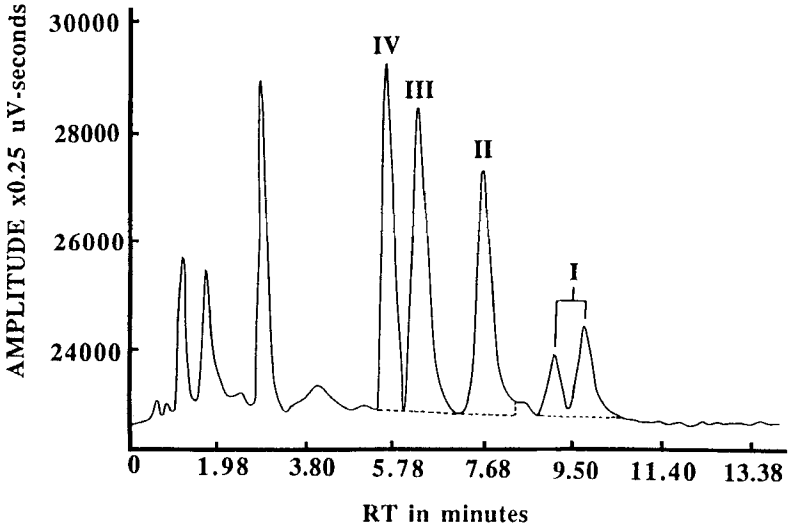

FIGURE 1: HPLC separation of syn and anti norgestimate (I), 3-keto-norgestimate (II), 17-deacetyl norgestimate (III), and norgestrel (IV); data based on UV absorption (254 nm). Column: Waters μBondapak C_{18}, 10 μm, 30 cm x 3.6 mm I.D.; Solvent system: methanol-water (80:20 v/v); Flow rate: 1 mL/min; pressure 1150 p.s.i.

TABLE 1
Recovery of Norgestimate Added to Blank Serum

Run No.	Quantity Added (pg/mL)	Quantity Measured (pg/mL)	Coefficient of Variation (%)	Recovery (%)	Coefficient of Determination (r^2)
1	50	49	21.0	98	0.9981
	100	88	6.5	88	
	250	251	17.1	100	
	500	546	8.2	109	
2	50	42	18.0	84	0.9967
	100	98	6.8	98	
	250	257	11.9	103	
	500	473	14.5	95	
3	25	28	25.0	112	0.9998
	50	51	10.9	102	
	100	89	10.0	89	
	250	242	8.8	97	
	500	499	5.8	100	
	1000	985	19.3	98	

TABLE 2
Recovery of Norgestimate in the Presence of 3-Keto-Norgestimate Added to Blank Serum

Concentration Ratio 3-Keto-Norgestimate	Quantity Added (pg/mL)	Quantity Measured (pg/mL)	Coefficient of Variation (%)	Recovery (%)	Coefficient of Determination (r^2)
1/5	25	24	8.7	96	0.9896
	50	59	0.5	82	
	100	104	3.4	104	
	250	246	0.3	98	
	500	610	9.3	122	
	1000	1021	13.9	102	
1/1	25	22	0.9	88	0.9856
	50	53	10.7	106	
	100	144	15.7	144	
	250	309	0.0	124	
	500	522	25.2	104	
	1000	---	---	---	
5/1	25	17	16.6	68	0.9928
	50	48	11.8	96	
	100	130	11.5	130	
	250	256	8.5	102	
	500	608	16.3	122	
	1000	1420	3.5	142	

DISCUSSION

The use of HPLC to impart specificity to existing RIA methods has been described for both steroids and peptides.[1-14] The method described in this report was developed for use in a bioavailability study of an oral contraceptive formulation containing norgestimate. Since one of the metabolites of norgestimate (3-keto-norgestimate) demonstrated significant cross-reactivity with the norgestimate antiserum, the separation of the two compounds was necessary in order to avoid an over-estimation of norgestimate serum levels. In addition, since the study was conducted in

premenopausal females, it was also necessary to separate any endogenous steroids from norgestimate prior to RIA. Inclusion of an HPLC separation in the procedure eliminates both the possibility of significant interference of one of the possible metabolites and by potentially cross-reactive endogenous steroids in serum that vary considerably during the normal menstrual cycle. Thus, accurate quantification of norgestimate would not be attainable without the HPLC separation step. This method has been recently applied successfully to serum samples obtained at various times after oral administration of tablets containing norgestimate. Using this procedure, nearly 50 samples could be conveniently extracted each day, and the extracts could then be loaded into the HPLC autosampler for overnight separation in preparation for RIA the following day(s).

In an effort to completely automate the method and optimize sample throughput, we are currently attempting at use a robotic work station to perform the extraction step. Thus, the procedure outlined is suitable for many applications involving routine sample analysis.

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