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Determination of onapristone and its N-desmethyl metabolite in human plasma or serum by high-performance liquid chromatography

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ABSTRACT

An automated reversed-phase high-performance liquid chromatographic method for the determination of the antiprogestin onapristone and its N-desmethyl metabolite in human plasma or serum is described Ultraviolet detection was performed at 315 nm, with a limit of detection of 1 ng/ml at a signal-to-noise ratio of 3 The intra- and inter-assay precision were ≤ 6% and ≤ 7%, respectively Onapristone and its N-desmethyl metabolite were stable in human plasma. The method was successfully applied to serum samples obtained from human volunteers after oral administration of onapristone

INTRODUCTION

Onapristone (I, ZK 98 299, Fig. 1) is a new compound that has shown anti-progestogenic properties in animals [1–3]. Clinical phase I and phase II studies are to be performed in order to test the efficacy of the antiprogestin in humans. A validated analytical method is a prerequisite for such pharmacokinetic studies.

In animal species onapristone is mono-N-demethylated to give the N-desmethyl metabolite (II, ZK 115 716; Fig. 1). This metabolism can also be expected in humans. The analytical method to be developed should therefore be able to detect both I and II.
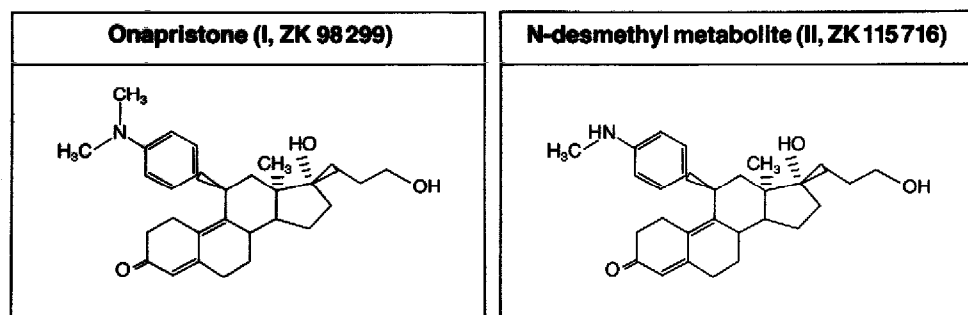

Fig. 1. Structures of onapristone (I) and its N-desmethyl metabolite (II).

High-performance liquid chromatography (HPLC) with column-switching allows direct injection of plasma and serum samples without additional off-line purification steps. This minimizes sample losses and, owing to the high reproducibility of the procedure, internal or external standardization is optional.

The aim in developing this method was to establish an automated HPLC procedure using column-switching for sample clean-up.

EXPERIMENTAL

Materials

Onapristone (I), 11β-(4-dimethylaminophenyl)-17α-hydroxy-17β-(3-hydroxypropyl)-13α-methyl-4,9(10)-gonadien-3-one and its N-desmethyl metabolite (II), 11β-(4-methylaminophenyl)-17α-hydroxy-17β-(3-hydroxypropyl)-13α-methyl-4,9(10)-gonadien-3-one, were synthesized in the laboratories of Schering (Berlin, F.R.G.).

The HPLC system consisted of a controlling unit (Model 680, Waters, Eschborn, F.R.G.), two pumps (Model 510, Waters), an autosampler with a thermostatted sample tray (ISS-100, Perkin-Elmer, Ueberlingen, F.R.G.,), a column-switching module (six-way valve, M & W, Berlin, F.R.G.) and a variable-wavelength UV detector (Spectroflow 773, Kratos, Karlsruhe, F.R.G.). The detector was connected via an interface to a mainframe computer (VAX 8810, Digital Equipment, Munich, F.R.G.) for data acquisition and evaluation.

The guard column (20 mm × 4.6 mm I.D.) was packed with LiChrosorb® RP 18 (particle size 15–25 μm). The analytical column consisted of a column cartridge system (RCM 8 × 10, Waters) with a Radial-Pak cartridge NovaPak® C_{18} (particle size 4 μm, 100 mm × 8 mm I.D.). In the investigations of the durability of the system, the cartridge system was sometimes replaced by stainless-steel columns (125 mm × 4.6 mm I.D.) filled with different materials.

Analytical-grade acetonitrile and methanol were obtained from Merck (Darmstadt, F.R.G.) and water was freshly distilled in an all-glass still. Solvents were degassed under water-jet vacuum, and the mobile phases were freshly prepared for each run.

Chromatographic procedure

Plasma samples were cooled to ca. 7°C in the autosampler, injected and transported by mobile phase I (acetonitrile–water, 5:95, w/w) to the guard column. During the washing period (flow-rate 1 ml/min) the constituents of the biological sample (e.g. plasma proteins, salts) were eluted to waste, while I and II were retained on the guard column. Meanwhile the analytical column was equilibrated with mobile phase II (acetonitrile–0.05 M phosphate buffer, pH 7.2, 40:60, w/w). After 3 min of washing the pneumatically driven six-way valve was switched to the alternative position. The retained compounds were then eluted from the guard column in back-flush mode with mobile phase II and separated on the

analytical column at a flow-rate of 2 ml/min. Both substances were detected by UV absorption at 315 nm. After a total of 13 min the six-way valve was switched back so that the guard column was equilibrated again with mobile phase I. The next analysis started after a total of 20 min.

Validation procedure

The linearity of the detector was investigated in the concentration range 0.49–1000 ng by means of twelve standard samples. The standard samples, with the substances dissolved in 50 μl of mobile phase II, were injected directly onto the analytical column. Each standard was analysed in triplicate.

Analytical recovery of I and II from the plasma was determined by a comparison of processed plasma samples with 1.95, 15.63, 125.0 and 1000 ng in 50 or 500 μl of plasma with the same amount of pure substance dissolved in 50 μl of mobile phase I. Each standard was analysed six times.

For calibration, twelve drug-free plasma samples (50 μl) were spiked with different amounts (0.49–1000 ng) of I and II prepared from three independently produced methanolic standard solutions.

The precision and accuracy of the method were assessed by repeated analysis (six independent assays) of control plasma samples (50 μl) that contained 5, 50 and 500 ng of I and II (inter-assay variance). Each control plasma was analysed six times within one assay (intra-assay variance).

In order to assess the influence of various column materials and compositions of mobile phase on retention time and peak shape, spiked plasma samples containing 125 ng of I and II were analysed under different conditions.

The stability of I and II was investigated in spiked plasma samples containing 5, 50 and 500 ng of the two substances in 50 μl of human plasma obtained from a young male volunteer. Each sample was analysed six times after different conditions of storage (seven days at $-20°C$ (control), seven days at 4–7°C, six days at $-20°C$ and one day at *ca.* 23°C, during seven days at $-20°C$ the samples were thawed over 1 h on five days).

Application of the method to human serum samples

Six female volunteers (age 65 ± 8 years) were treated with increasing doses of I during a clinical phase I study (tolerance of I). Ethical approval was received from an Institutional Review Board of Schering according to the regulation of the declaration of Helsinki (Venice Amendment, 1983). Each woman received placebo, 25, 50, 100, 200 and 400 mg of the antiprogestin as a single oral dose at weekly intervals. Serum samples were collected prior to and 1, 2, 4, 8 and 12 h after administration. Depending on the expected concentrations of the drug in the serum, sample volumes between 50 and 500 μl were analysed in a randomized order together with calibration and control samples.

The applicability of the validated method on serum was checked by simultaneous analysis of serum and plasma samples spiked with known amounts of I and II.

RESULTS

Linearity of detection range

In the range 0.49–1000 ng, a linear regression line was obtained for I and II ($r^2 \geq 0.9999$).

Recovery

In comparison with the methanol-diluted drug standards the recoveries of I and II from plasma were 93–123 and 91–108%, respectively. The recoveries of both substances were independent of the injected plasma volume (see Table I).

Calibration curve and limit of detection

Injection of drug-free plasma samples revealed no interfering background signals (see Fig. 2). The limit of detection (signal-to-noise ratio 3) of pure drug standards was 0.49 ng on-column, corresponding to 1 ng/ml (500 μl) and 10 ng/ml (50 μl) for both substances.

For the analysis of plasma samples the calibration curves of the two substances were divided into two parts. Best-fit calibration curves were obtained for an injection volume of 50 μl in the ranges of 0.01–0.3 and 0.3–10 μg/ml, and for an injection volume of 500 μl in the ranges of 1–31 and 31–1000 ng/ml (I, $r^2 \geq 0.979$; II, $r^2 \geq 0.994$).

Precision

Repeated analysis ($n = 6$) of control plasma samples containing low, medium and high amounts of I and II revealed coefficients of variation (C.V.) of intra-assay precision in the range 0.6–5.9 and 0.7–4.7% for the drug and its metabolite,

TABLE I

ANALYTICAL RECOVERY OF THE ANTIPROGESTIN ONAPRISTONE I AND ITS N-DES-METHYL METABOLITE II FROM SUPPLEMENTED DRUG-FREE PLASMA SAMPLES

Results are given as a percentage of the nominal value (mean ± S.D., $n = 6$).

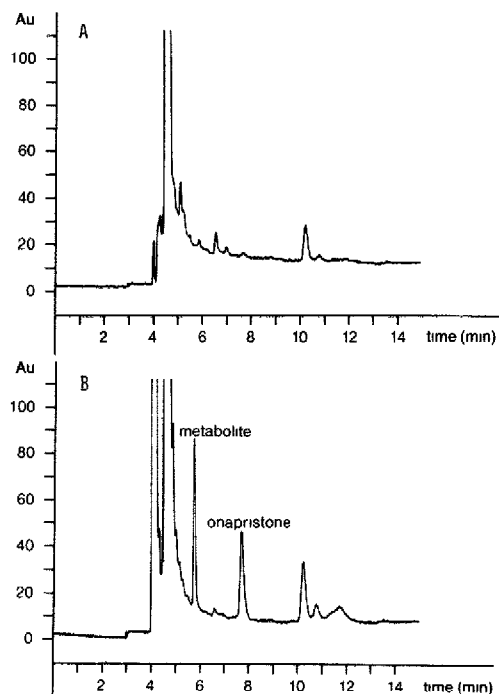| Compound	Amount injected on-column (ng)	Recovery (%)	
		50 μl injected	500 μl injected
I	1.95	119 ± 11	123 ± 12
	15.6	111 ± 10	101 ± 4
	125	97 ± 1	104 ± 7
	1000	93 ± 1	107 ± 7
II	1.95	91 ± 6	102 ± 10
	15.6	102 ± 10	92 ± 6
	125	94 ± 1	102 ± 7
	1000	93 ± 1	108 ± 8

Fig. 2. (A) Chromatogram (λ = 315 nm) of a 500-μl drug-free plasma sample (B) Chromatogram of a 500-μl drug-free plasma sample spiked with 16 ng each of onapristone and its N-desmethyl metabolite The elution times of 5.8 and 7.7 min include 3 min of sample enrichment on a separate guard column (mobile phase, acetonitrile–phosphate buffer)

respectively (see Table II). The C.V. values of inter-assay precision varied from 1.4 to 6.6% (I) and from 2.7 to 5.0% (II).

TABLE II

PRECISION OF THE DETERMINATION OF I AND II IN 50-μl PLASMA SAMPLES

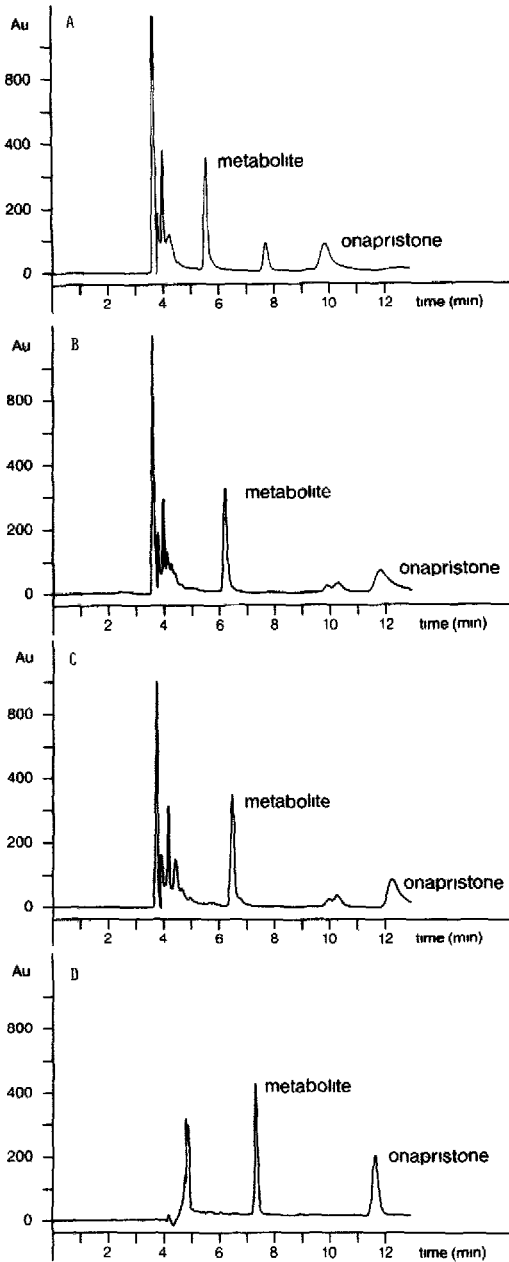| Compound	Concentration (μg/ml)	Intra-assay precision (%)	Inter-assay precision (%)
I	0.1	5.9	6.6
	1	2.3	1.4
	10	0.6	5 2
II	0.1	4.7	3.9
	1	1.6	2 7
	10	0.7	5 0

Fig. 3. Chromatograms of plasma samples spiked with 125 ng of onapristone and of its N-desmethyl metabolite after analytical separation on (A) ODS Hypersil (5 μm), (B) Spherisorb ODS II (5 μm), (C) LiChrosorb RP 18 (5 μm) and (D) NovaPak C_{18} (4 μm) columns The elution times of both substances include 3 min of sample enrichment on a separate guard column.

Accuracy

The accuracy of control plasma samples calculated from the deviation of the measured amount from the nominal value of each substance indicated a inter-assay variation in the range from − 16.1 to 14.3% for I and from 0.6 to 24.6% for II.

Durability

The suitability of different reversed-phase materials was tested using ODS Hypersil® (particle size 3 and 5 μm), Spherisorb® ODS II and LiChrosorb RP 18 (particle size 5 μm) and NovaPak C$_{18}$ (particle size 4 μm). Of these materials only NovaPak C$_{18}$ produced symmetrical peaks of I and II, if large volumes (> 0.2 ml) of spiked plasma samples were injected directly onto the guard column (see Fig 3).

Variation of the pH of the mobile phase between pH 5 and 7.5 had no influence on the shapes of the peaks of the two substances. Changing the solvent from methanol to acetonitrile reduced the pressure and slightly increased the separation efficiency.

Stability of I and II in plasma

In order to assess the stability of I and II in human plasma, the contents of spiked plasma samples were analysed under different conditions of storage and treatment. Neither I nor II revealed instability during storage at 23°C over 24 h or storage in the refrigerator over seven days. Even freezing and thawing five times had no influence on the stability of the two compounds (see Table III).

TABLE III

IN VITRO STABILITY OF I AND II IN HUMAN PLASMA UNDER DIFFERENT STORAGE CONDITIONS

Results are given as recovery in relation to seven days of storage at − 20°C (n = 4) for a plasma volume of 50 μl.

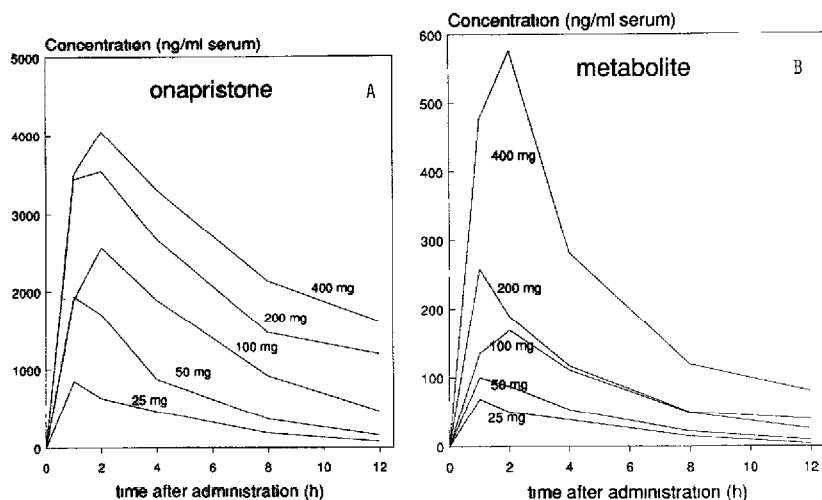| Compound	Concentration (μg/ml)	Recovery (%)		
		Seven days +4–7°C	Six days − 20°C, one day +23°C	Five times freezing–thawing
I	0 1	98.8	100 1	102 2
	1	96.3	101 4	100 0
	10	98.6	99.9	100 2
II	0.1	97.6	95.8	93.4
	1	98.3	98.1	101 7
	10	98.7	99.9	99 7

Fig. 4. Serum levels of (A) I and (B) II after oral administration of five different doses of I to six female volunteers Results are plotted as mean values.

Serum levels of I and II

The concentrations of the drug and its metabolite were determined in 180 serum samples. No differences were observed between serum and plasma samples spiked with known amounts of I and II. Depending on the dose administered the maximum serum levels of I and II increased from 933 ± 266 to 4435 ± 901 ng/ml and from 83 ± 24 to 670 ± 134 ng/ml, respectively (see Fig. 4). The limit of quantification was defined as 5 ng/ml for an injection volume of 500 μl.

DISCUSSION

I and II were measurable over a wide range, between 1 ng/ml and 10 μg/ml. Independent of the plasma volume injected, the recovery of the antiprogestin and its metabolite form plasma was generally between 90 and 110%, allowing simultaneous analysis of different volumes within one assay.

The intra- and inter-assay precisions of the method were lower than 7%. The accuracy was sufficient. Only at the lowest concentrations a slightly elevated determination of up to 15% might be possible.

Changes of the column materials influence the analysis considerably, especially at low concentrations of I and II. Modification of the mobile phase has only a minor influence on the determination of the two compounds. Both substances are stable in human plasma.

Simultaneous analysis of spiked plasma and serum samples indicated that the method can also be applied to serum. Therefore, in the present investigation, the concentrations of the drug and its metabolite were determined in a number of

serum samples obtained from female volunteers who were treated with increasing oral doses of I.

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