QUANTITATION OF RU 486 IN HUMAN PLASMA BY HPLC AND RIA AFTER COLUMN CHROMATOGRAPHY

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ABSTRACT

Chromosorb^{R} column chromatography was used for separation of RU 486 from its immunologically cross-reacting metabolites prior to quantitative analysis by radioimmunoassay (RIA) or high-performance-liquid chromatography (HPLC). The results of the two assay methods were in good agreement with each other (r=0.99, n=29). The retention time of RU 486 in our HPLC system was 2.5 min.

Plasma concentrations of RU 486 were measured by HPLC up to 48 h following single oral administration of 100, 400, 600 and 800 mg of RU 486 to female volunteers. The plasma peak concentrations (2.0 - 2.5 μg/ml) were reached within the first hour. After redistribution, the plasma concentrations of RU 486 were not significantly affected by the doses studied but remained in the same range throughout the 48 hours. The plasma half-life between 24 and 48 hours was 27 hours or more.

We conclude that HPLC is valuable in studies on the metabolism and pharmacokinetics of RU 486, but a less laborious RIA method after Chromosorb^{R} column chromatography is suitable and gives reliable results in large-scale clinical studies.

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INTRODUCTION

RU 486 is a new 19-norsteroid derivative with potent anti-progestational (1) and anti-glucocorticoid activity (2,3). A preliminary clinical study showed that RU 486, given in early pregnancy, resulted in abortion in 9 of 11 women (4). It also induced menses at the midsecretory phase of the cycle (4). Antiprogestational effect of RU 486 is mediated via its binding to the human progesterone receptor with higher affinity than progesterone (5). Results of studies with RU 486 as a new antifertility agent are very promising.

Since the metabolites of synthetic steroids are often biologically active, their concentrations in blood can also have clinical significance. We have utilized a human plasma pool, collected after oral intake of RU 486, as a source of a mixture of RU 486 and its metabolites. The putative metabolites of RU 486 were separated by thin layer chromatography and characterized by RIA, UV-spectrophotometry and HPLC.

The need for a specific and reliable assay method in evaluation of RU 486 is obvious. The RIA for RU 486 developed by Salmon and Mouren (6) is nonspecific for its metabolites (7). Our assay methods developed for RU 486 utilize Chromosorb® column chromatography to eliminate those putative metabolites cross-reacting in the direct RIA. The quantitative analysis of RU 486 is performed by HPLC or RIA. Plasma concentrations of RU 486 were measured for 48 hours after oral administration of 100, 400, 600 and 800 mg of RU 486.

MATERIALS AND METHODS

Chemicals: RU 486 (17β-hydroxy-11 β-(4-dimethylaminophenyl)-17α-(1-propynyl)-estra-4,9-dien-3-one), (6,7-^{3}H)-RU 486 (specific activity 37 Ci/mmol) and the corresponding antibody were provided by the Roussel-Uclaf Research Center (Romainville, France). n-Hexane, ethyl acetate, ethylene glycol, diethyl ether, methanol, triethanolamine, chloroform and acetone were purchased from Merck (Darmstadt, West Germany). Chromosorb® W-NAW 60-80 Mesh was from Sigma (St. Louis, Missouri, USA) and Macherey-Nagel (Duren, West Germany); Kieselgel 60 F 264 and the octadecyl reversed phase HPLC column Hibar LiChrosorb RP-18 (250 mm x 4 mm internal diameter) were from Merck.

Laboratory equipment: HPLC system consisted of Solvent programmer Model 660, two chromatography pumps, Model M-6000 A and liquid chromatography injector Model U 6 K from Waters Associates Inc. (Milford, Massachusetts, USA). The spectrophotometric detector LC-55B and deuterium power supply for the HPLC system were purchased from Perkin-Elmer (Oak Brook, Illinois, USA); the recorder was a Servogor RE 511 from Goerz Electric (Vienna, Austria).

Spectrophotometric studies were performed with a Graphicord UV-240 and Option program, interface OPI-2 from Shimadzu (Kyoto, Japan).
A liquid scintillation counter 1212 Minibeta from Wallac (Turku, Finland) and Teleprinter 43 from Teletype Corporation (Skokie, Illinois, USA) were used in our radioimmunoassay studies.

Plasma samples: Plasma samples were collected from healthy female volunteers, aged from 25 to 32 years, in the midluteal phase following oral ingestion of 100, 600 and 800 mg of RU 486 by five subjects and 400 mg by four subjects (50 mg tablets provided by Roussel-Uclaf, Paris, France). In the group receiving 100 mg of RU 486 blood samples were collected at -1/2, 0, 1, 2, 4, 6, 24 and 48 hours. Samples were collected at -1/2, 0, 1, 2, 4, 6, 10, 24 and 48 hours in groups receiving 400, 600 and 800 mg, these groups were part of RU 486 tolerance study (8).

Detection of immunoreactive metabolites was carried out as follows:

* Diethyl ether extraction of a plasma pool collected from female volunteers after oral intake of 200 or 400 mg of RU 486.

* Thin layer chromatography (TLC) of standard RU 486, extracts of blank plasma and female plasma pool on Kieselgel 60 F 264 with chloroform:acetone [90:10] (v/v).

* Elution of 5 mm TLC-slices with 2 ml of ethanol.

* RIA of the eluates as described below.

* UV-absorption spectra of the RIA-positive eluates.

* HPLC (Hibar LiChrosorb RP-18 column) fractionation of the immunoreactive eluates with methanol:water [90:10] (v/v); flow rate 1 ml/min.

* UV-absorption spectra of the HPLC-fractions.

Separation of RU 486 from its metabolites on Chromosorb^{R} columns:
Column chromatography was performed by packing Pasteur pipettes under vibration with 3 ml of Chromosorb^{R} W-NAW 60-80 mesh/20% ethylene glycol (w/w).
A patient plasma sample, blank plasma pool containing RU 486 and

blank plasma containing ^{3}H-RU 486 were applied to the columns, left for 30 minutes and then eluted as follows: (I) 5 ml of ethyl acetate:n-hexane [5:95] (v/v), (II) 6 ml of ethyl acetate:n-hexane [30:70] (v/v), (III) 5 ml of ethyl acetate:n-hexane [75:25] (v/v) and (IV) 5 ml of ethyl acetate. The eluates were assessed by RIA as described below or the radioactivity was counted.

Quantitation of RU 486:

I Chromosorb^{R} column chromatography: 0.2 - 0.4 ml of plasma/serum was applied on the column described above, left for 30 min, eluted with 5 ml of ethyl acetate:n-hexane [5:95] (v/v) and evaporated under nitrogen until dry. For the analysis of plasma samples a standard curve was prepared by running plasma pools (prepared by addition of known amounts of RU 486 into blank plasma) through the columns.

IIa For HPLC of RU 486 samples were redissolved into appropriate volume of the HPLC eluent used routinely: methanol:water: triethanolamine [90:10:0.05] (v/v/v), pumped at 1.5 ml/min; the injection volume was 100 µl.

IIb For RIA of RU 486 samples were redissolved into appropriate volume of 0.1 M phosphate-gelatin buffer containing 20% methanol (v/v). RIA was performed as described below.

Evaluation of the Chromosorb^{R}-HPLC method: Plasma pools, containing 0.25, 1.0 and 5.0 µg of RU 486/ml were prepared for the recovery studies. Five 0.2 and 0.4 ml samples of each pool were chromatographed in the Chromosorb^{R} columns. The eluates were redissolved into corresponding volume of methanol. Fifty microlitres was evapor ted until dry, redissolved into buffer used for RIA as described below. The rest was evapor ted until dry and redissolved into HPLC eluent. RU 486 concentrations were measured both by RIA and the routine HPLC system and compared with dilution series to assess the recovery.
The sensitivity of the routine HPLC method was controlled by assaying an RU 486 dilution series. With the injection volume of 100 µl, the amount of RU 486 injected varied from 30 ng to 2.0 ng.

Radioimmunoassay of RU 486 without chromatography: A phosphate buffer containing 0.1% gelatin (w/v) was prepared by dissolving 9 g NaCl, 1 g sodium azide and 1 g gelatin in 1 liter of 0.1 M phosphate buffer, pH 7.0 and stored at + 4°C.
The antiserum against RU 486 was donated by Roussel-Uclaf, diluted 1/100 (v/v) into phosphate-gelatin buffer and stored at - 20°C.
A stock solution of RU 486 at 1.0 mg/ml was stored at +4°C. For the standard curve, solutions of RU 486 were prepared just before use by pipetting a dilution series containing 2 - 2000 pg of RU 486 in ethanol per plastic test tube. The standards were evaporated until

dry and dissolved in 100 µl of phosphate-gelatin buffer containing 20% methanol (v/v).

Tritiated RU 486 was stored at -20°C in toluene. Prior to use (6,7-^{3}H)-RU 486 was purified using the Chromosorb^R-method followed by dilution in phosphate-gelatin buffer containing 0.025% Triton X-100 (w/v) (Koch-Light Laboratories Ltd, Colnbrook, Bucks, England) at a concentration of 50,000 counts per 1 ml.
A charcoal suspension was prepared, composed of 0.25% charcoal (w/v) (Norit A, from Sigma) and 0.025% dextran T70 (w/v) (Pharmacia, Uppsala, Sweden) in phosphate-gelatin buffer.
Scintillation fluid YA-riatuike (70% pseudochumene) was obtained from Yliopiston Apteekki (Helsinki, Finland).
Radioimmunoassay was performed according to Salmon and Mouren (6) as follows:
Plasma samples of 0.1 ml were diluted with 0.4 ml of physiological saline containing 0.025% Triton X-100 (w/v). The samples were vortex-mixed and extracted once with 5 ml of diethyl ether for 10 minutes. The aqueous phases were frozen in -70°C cold ethanol and the extracts were evaporated to dryness with nitrogen in a +40°C water bath. The samples were dissolved in 0.1 ml phosphate-gelatin buffer containing 20% methanol. They were then mixed with 0.1 ml of antibody diluted in phosphate-gelatin buffer and left for 30 minutes at room temperature. Tritiated RU 486 (0.1 ml) was added, vortexed and left to incubate overnight in an ice-bath. Ice-cold charcoal suspension (0.5 ml) was added together with 0.1 ml of 0.5% phosphate-gelatin buffer (w/v), left for 5 min on ice and then centrifuged for 10 minutes at 3000 x g. A Finnfuge centrifuge (Labsystems Oy, Helsinki, Finland) was used. Supernatants were transferred to polyethylene counting vials together with 2.5 ml of scintillation fluid and were counted for 5 minutes.

Statistical analysis: Welch paired t-test was used for statistical analysis. The half-life of RU 486 was calculated graphically from the RU 486 concentrations measured at 24 and 48 hours.

RESULTS

Figure 1 shows the TLC of RU 486 standard, as well as the extracts of blank plasma and plasma pools obtained by combining samples collected from female volunteers four or forty-eight hours after oral administration of 200 or 400 mg of RU 486. RIA was performed on the eluates of the sliced TLC in order to find the cross-reacting steroids. Four distinguishable spots were found with the RIA, viz. authentic RU 486 with an Rf-value of 0.56, two UV-visible spots with Rf-values of 0.38 and 0.19 and a spot with an Rf-value of 0.11. UV-absorption spectra of the 0.56, 0.38 and 0.19 spots revealed absorption maximum at 304 nm.
The UV-absorption spectrum of RU 486 had absorption maxima at 304 and 262 nm. By using a wave-length of 304 nm in the fractionation HPLC

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