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Simultaneous determination of rivanol and mifepristone in human plasma by a HPLC-UV method with solid-phase extraction

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Abstract

A HPLC method with UV detection was developed and validated for the simultaneous determination of rivanol and mifepristone in human plasma. Norethisterone was used as the internal standard. Separation was performed by a C18 reversed-phase column maintained at 20 °C. The mobile phase was a mixture of methanol–acetonitrile–0.05% sodium dodecylsulfonate in a 0.05 M phosphate buffer with the pH adjusted to 3.0 (30:30:40, v/v/v) at a flow rate of 0.8 ml/min. Dual wavelength mode was used, with mifepristone monitored at UV 302 nm, while rivanol and norethisterone at 272 nm. A reliable biological sample pre-treatment procedure by means of solid-phase extraction was used, which allowed to obtain good extraction efficiency (>93%) for both of the analytes and the internal standard. The calibration curves were both linear with the correlation coefficient *r* equal to 0.9999. For rivanol, the assay gave CV% values for precision always lower than 7.8% and mean accuracy values higher than 95.3%. As to mifepristone, precision was always lower than 10.1% and mean accuracy values were higher than 93.8%. The limit of detection for the assay of rivanol and mifepristone was 1.1 and 3 ng/ml, respectively. The method is simple, sensitive and accurate, and allow for simultaneous determination of nanogram levels of rivanol and mifepristone in human plasma. It could be applied to assess the plasma level of rivanol and mifepristone in women undergoing polypharmacy with the two drugs.

Keywords: Rivanol; Mifepristone; Human plasma; High performance liquid chromatography; Solid-phase extraction

1. Introduction

Rivanol, as shown in Fig. 1, is commonly used as a drug for second trimester termination of pregnancy by injecting into the amniotic cavity in China, which proved to be associated with the lowest rate of complications [1]. Due to its advantages, this method has attracted a lot attention from researchers in the Western world too [2]. Mifepristone (Fig. 1) has been world-widely used for terminating pregnancy as a competitive inhibitor of progesterone since synthesized in 1980s [3]. Initially, the two drugs were used solely. In recent 15 years, combined usage of rivanol and mifepristone to treat midtrimester abortion was widely experimented in China, because it could effectively shorten the course of abortion, alleviate the pain, improve the success rate, reduce placenta embryolemma remaining, and decrease the occurrence rate of infection [4]. In the passed 10 years, at least 500 Chinese literatures focused on this subject. Therefore, simultaneous determination of rivanol and mifepristone in human plasma is important in studying pharmacokinetics, the appropriate doses and possible pharmacological interactions of the two drugs when the young women undergo polypharmacy with them.

However, to our best knowledge, no analytical method has been described in the literature which simultaneously determines these two analytes, even though several methods exist, which separately determine either rivanol [5–11] or mifepristone [12–19]. Obviously, it is discommodious to determine rivanol and mifepristone separately for each sample under two different HPLC conditions. In the present study, we developed a simple, sensitive and accurate HPLC method, which

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could simultaneously detect nanogram levels of rivanol and mifepristone in human plasma. Besides the one of simultaneous determination of rivanol and mifepristone in human plasma, advantages and novelty of the proposed method relative to existing methods separately determining rivanol and mifepristone are either its advancement in technique and instrumentation, i.e., using HPLC method instead of titration, fluorescence spectra, catalytic photokinetic, spectrophotometric and electroanalytical method ones, or its simplified and optimized experimental procedures, e.g. the solid-phase extraction step procedure instead of a liquid–liquid one, addition of a proper internal standard for increased accuracy and precision instead of external standard method and lower limits of detection and quantification.

2. Experimental

2.1. Chemicals and reagents

Rivanol was purchased from Sigma–Aldrich (St. Louis, MO, USA). Mifepristone and norethisterone (Fig. 1) used as the internal standard (IS), were kindly donated by XianJu Pharmaceutical Co. Ltd. (Zhejiang, China), with their purities reported as ≥99%. HPLC-grade methanol and acetonitrile were purchased from Tedia Company, Inc. (Fairfield, OH, USA). Ultrapure water (18 MΩ) was obtained by a HB-RO/10 deionization ultrapure system (Huibang Co., Hangzhou, Zhejiang, China). All other chemicals and reagents were of analytical grade available commercially.

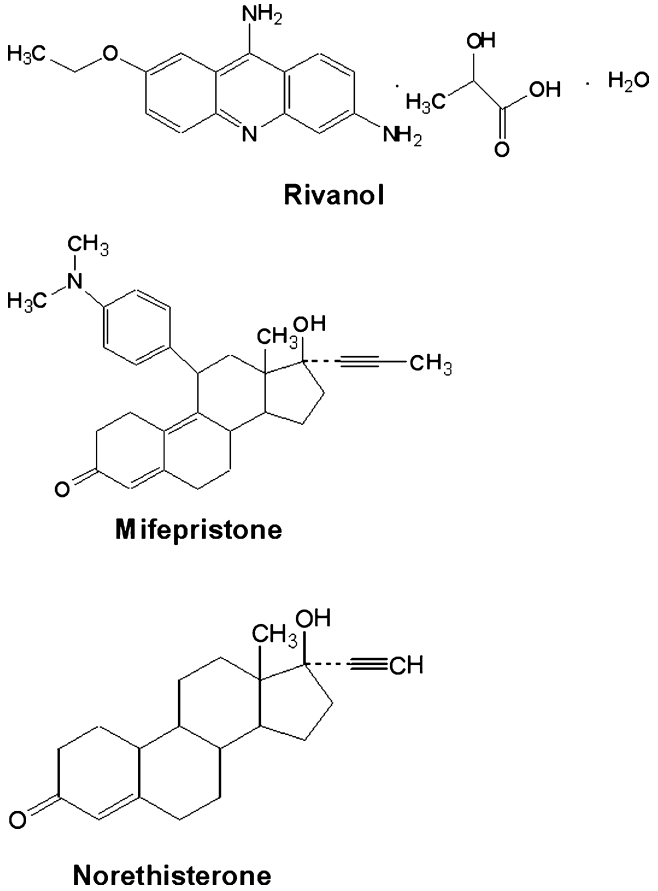

Fig. 1. Chemical structures of rivanol, mifepristone and norethisterone.

2.2. Apparatus and chromatographic conditions

Chromatographic analyses were performed using a Shimadzu HPLC system (Nakagyo-ku, Kyoto, Japan) consisted of two Shimadzu LC-10AT$_{VP}$ pumps, a Shimadzu CTO-10A column oven, a Shimadzu SPD-10A$_{VP}$ UV detector, a Shimadzu DGU-20A3 on-line solvent degasser and a Rheodyne 7725i manual injector (Cotati, CA, USA) with final volume loop of 20 μl. The experimental parameters of the HPLC system, including monitoring wavelength, flow rate, concentration of the mobile phase and column temperature, were directly controlled by the control panel of each instrument unit. Hardware interface for data acquisition and integration was provided by the N2000 computer software package (Zhida Co., Hangzhou, Zhejiang, China). The analytical column used was a Shimadzu Shim-Pack VP-ODS reversed-phase column (150 mm × 4.6 mm i.d., 4.6 μm particle size) protected by a Shimadzu Shim-Pack guard column (10 mm × 4.6 mm i.d., 4.6 μm particle size). The mobile phase was a mixture of methanol–acetonitrile–0.05% sodium dodecylsulfonate in a 0.05 M phosphate buffer with the pH value adjusted to 3.0 (30:30:40, v/v/v). The flow rate was set at 0.8 ml/min. The wavelengths of UV detector were operated at 302 and 272 nm by using dual wavelength mode, with the former for mifepristone and the latter for rivanol and norethisterone. The column temperature was regulated at 20 °C.

2.3. Preparation of stock and working solutions

Stock solutions of rivanol, mifepristone and norethisterone (IS) were prepared at the concentration of 1 mg/ml by dissolving suitable amounts of each pure substance in methanol and stored in cleaned brown glass bottles. They were stored at −20 °C under dark condition until they were used for preparing working solutions by adding the appropriate volume of water to prevent precipitation of proteins when added to the plasma. Assessed by HPLC, these stock solutions were stable for at least 15 days. Working solutions of different concentrations were prepared from above-mentioned stock solutions afresh just prior to use.

2.4. Preparation of calibration standard and quality control (QC) samples in plasma

Calibration standards in plasma at the concentration of 3, 5, 10, 50, 100, 500, 1000 ng/ml were prepared by spiking appropriate aliquots of working solutions of rivanol and mifepristone, as well as 0.1 ml of norethisterone at the concentration of 10 μg/ml (final 1 μg), to 1 ml of blank human plasma. QC samples were also prepared as above at concentration of 10, 100, 1000 ng/ml. These calibration standards and QC samples were then treated using the procedure described in Section 2.6.

2.5. Human plasma sampling

Sample collection was carried out in the Affiliated Hospital of Anhui Research Institute for Family Planning located at Hefei

in China. Five healthy women, 23–28 years of age, who were in the second trimester of pregnancy at the time of sampling, were chosen as volunteers. Because they did not want a baby at that time, so they volunteered to abort by combined usage of rivanol and mifepristone in the government hospital, and they volunteered to join in this study too. Prior to their participation, each woman signed an informed consent document.

Each volunteer received a single intra-amniotic injection of 100 mg rivanol and a single oral dose of 150 mg mifepristone simultaneously at 0 h. When the abortion was accomplished, with the time ranging from 28.3 to 36.2 h, samples were collected by venipuncture into a glass tube containing heparinate anticoagulant. The plasma was separated by centrifugation for 5 min at 3000 × *g* at room temperature, and then it was harvested and stored at −20 °C until analysis.

2.6. Solid-phase extraction (SPE) procedure

All samples, including blanks, standards, QCs and unknowns, were extracted using solid-phase extraction cartridges (Oasis HLB 1 ml, 30 mg), which were purchased from Waters (Milford, MA, USA). Each cartridge was equilibrated by 1 ml of methanol, and then conditioned by 1 ml of ultrapure water. One milliliter of 0.05% sodium dodecylsulfonate in a 0.05 M phosphate buffer with the pH value adjusted to 3.0 was added into the plasma sample and vortex-mixed for 5 min. Thereafter, the mixed solution was loaded onto and passed through the cartridge without lab vacuum. After washed with 1 ml 5% methanol aqueous solution, the analytes were eluted with 3 ml 100% methanol and collected in clean glass tubes. The eluent was evaporated to dryness under −0.08 MPa lab vacuum at 37 °C, and the extraction residue was reconstituted in 100 μl of mobile phase just prior to injection into the HPLC system. About 50 μl aliquot was manually injected to insure that the 20 μl loop of the injector was totally full.

3. Results and discussion

3.1. Method development

Due to the strong polar groups in it, the chromatographic peak of rivanol would be tailing severely if water was used directly in the mobile phase. When 0.05% sodium dodecylsulfonate aqueous solution at pH 3 was used instead of pure water, rivanol would change to the ionized form in acidic environment and combine with dodecylsulfonate group acting as the counterion, to form an ion-pair that exhibited very good retention characteristic on the C18 column. The chromatographic peak of rivanol was then very symmetrical as shown in Fig. 2. In addition, strong molecular polarity of rivanol could not allow itself to be adsorbed effectively in aqueous solution by the sorbent of the Oasis HLB cartridge, macroporous copolymer [poly(divinylbenzene-co-*N*-vinylpyrrolidone)]. Therefore, 1 ml of 0.05% sodium dodecylsulfonate aqueous solution at pH 3 was added into the plasma sample to form an ion-pair, thus very good retention characteristic of rivanol would be achieved on the Oasis HLB cartridge.

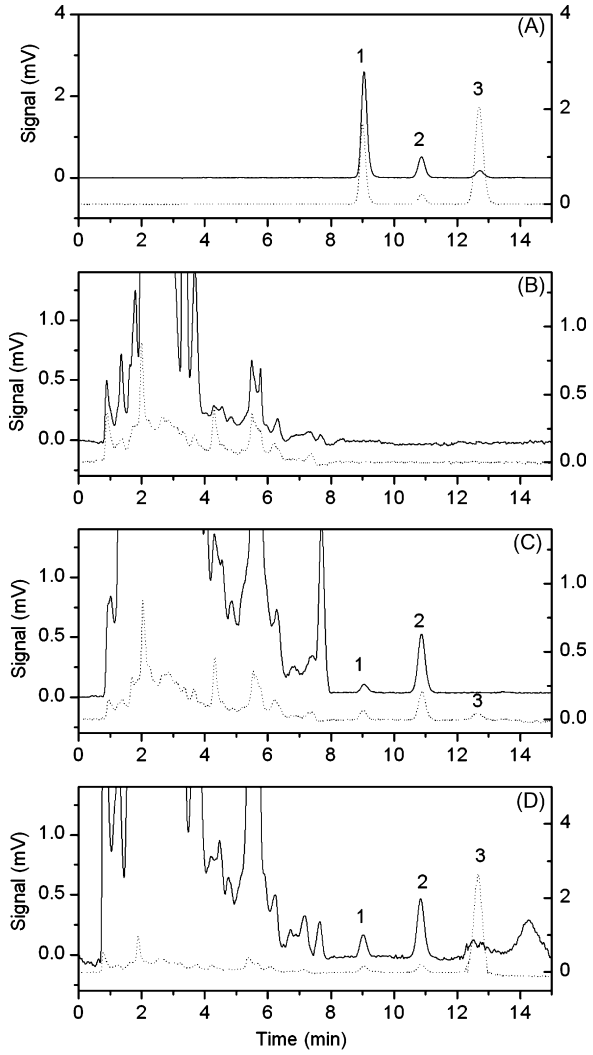

Fig. 2. HPLC chromatograms of rivanol and mifepristone. (A) Mobile-phase solution spiked with 1000 ng/ml of rivanol, mifepristone and IS, (B) blank human plasma, (C) human plasma spiked with 3 ng/ml of rivanol, 3 ng/ml of mifepristone and 1000 ng/ml of IS, (D) a human plasma collected when the abortion was accomplished, the concentrations of rivanol and mifepristone were 8.4 and 126 ng/ml, respectively. Peak 1, rivanol; peak 2, IS; and peak 3, mifepristone.

The wavelengths of maximum absorbance for rivanol, mifepristone and norethisterone are 272, 302 and 240 nm, respectively. As the Shimadzu SPD-10A$_{VP}$ UV detector has only two wavelength channels, 272 nm for rivanol and 302 nm for mifepristone were selected to ensure good sensitivities of the two drugs. Norethisterone was detected at 272 nm, as higher sensitivity would be achieved than at 302 nm. Its concentration was selected as 1 μg/ml to acquire proper peak areas.

3.2. Extraction efficiency

Extraction efficiencies were calculated by comparing the mean peak areas of three extracted QC plasma samples ($n = 3$) to those of three non-extracted samples ($n = 3$) of the same concentration. Extracted plasma samples were subjected to extraction, drying and reconstitution as described above. Non-extracted samples were prepared in methanol, and subjected to drying and

Table 1
Extraction efficiency for rivanol, mifepristone and IS (mean ± SD, $n = 3$)

	Rivanol (mean ± SD)			Mifepristone (mean ± SD)			IS (mean ± SD)
	10 ng/ml	100 ng/ml	1000 ng/ml	10 ng/ml	100 ng/ml	1000 ng/ml	1000 ng/ml
Non-extracted peak area	4108 ± 161	34523 ± 879	357663 ± 20988	3943 ± 277	36663 ± 1984	384935 ± 23642	8106 ± 58
Extracted peak area	4045 ± 65	35001 ± 1362	335478 ± 32146	4011 ± 126	38801 ± 1513	365913 ± 12988	8418 ± 48
Extraction efficiency (%)	98.5	101.4	93.8	101.7	105.8	95.1	103.8

reconstitution without the extraction procedure. The extraction efficiency was calculated as (peak area found after extraction × 100)/(peak area without extraction). As shown in Table 1, extraction efficiency of rivanol and mifepristone varied in the range of 93.8–101.4% and 95.1–105.8%, respectively. And the internal standard extraction averaged 103.8%. High extraction efficiency of rivanol, mifepristone and IS suggested that little was lost during the extraction process.

3.3. Selectivity and specificity

Negative human plasma samples of the five volunteers collected at 0 h were analyzed, and the eventual background in the area of the rivanol/mifepristone/IS retention time was used to evaluate the selectivity of this method. The specificity was assessed by comparing the signal for blank human plasma extract with the response of an extract spiked with rivanol, mifepristone and IS and those of plasma samples collected from the volunteers. A series of typical chromatograms are shown in Fig. 2. The left axis is for the chromatograms recorded at 272 nm indicated as the solid lines, while the right axis is for the ones at 302 nm indicated as the dotted lines. Fig. 2(A) shows the chromatograms of rivanol, mifepristone and IS in the mobile phase. The concentration of all of them was 1000 ng/ml. The three peaks of the two analytes and the IS were resolved thoroughly. The retention times for rivanol, mifepristone and IS were 9.05 ± 0.09, 12.64 ± 0.13 and 10.86 ± 0.12 min ($n = 20$), respectively. Fig. 2(B) shows the chromatograms of blank plasma, which illustrated that there were no interfering peaks coming at the elution times for rivanol, mifepristone or IS. Although there were characteristic peaks for the biological matrix, they did not interfere with the peaks for the two analytes or the IS. Fig. 2(C) shows the chromatogram of standard plasma sample spiked with 3 ng/ml of rivanol, 3 ng/ml of mifepristone and 1000 ng/ml of IS, in which the peaks for 3 ng/ml of rivanol and 3 ng/ml of mifepristone could be identified easily and accurately. Fig. 2(D) shows the chromatogram of a human plasma sample collected as described in Section 2.5, and the concentrations of rivanol and mifepristone in plasma were 8.4 and 126 ng/ml, respectively.

3.4. Linearity and sensitivity

The linearities were checked by using the internal standard calibration curves. Peak area ratio of rivanol was determined by comparing the peak area of rivanol with various concentrations at 272 nm to the peak area of IS with the concentration of 1000 ng/ml at 272 nm. And peak area ratio of mifepristone was determined by comparing the peak area of mifepristone with various concentrations at 302 nm to the peak area of IS with the concentration of 1000 ng/ml at 272 nm. The calibration standard curves for rivanol were constructed by plotting mean ($n = 3$) peak area ratios of analyte to IS *y versus* rivanol concentrations in plasma x (ng/ml) with the concentrations of 0, 3, 5, 10, 50, 100, 500 and 1000 ng/ml. And that for mifepristone were done with the concentrations of 0, 10, 50, 100, 500 and 1000 ng/ml. For rivanol, the linear regression equation was $y = 0.0405x + 0.0795$ with the correlation coefficient r equal to 0.9999. And for mifepristone, the linear regression equation was $y = 0.0462x + 0.0538$ with the correlation coefficient r equal to 0.9999.

Based on 3/1 of the signal-to-noise ratio, the limit of detection (LOD) for the assay of rivanol and mifepristone was about 1.1 and 3 ng/ml, respectively. And based on 10/1 of the signal-to-noise ratio, the limit of quantitation of rivanol and mifepristone was about 3.6 and 10 ng/ml, respectively [20]. The LOQs of the assay were validated by five QC samples prepared and treated as described above, with rivanol at the concentration of 3 ng/ml and mifepristone at the concentration of 10 ng/ml. Expressed by mean ± standard deviation ($n = 5$), the concentration of rivanol was 3.38 ± 0.44 ng/ml, with the accuracy 112.7% and relative standard deviation (RSD) 13.0%. And that of mifepristone was 10.67 ± 1.08 ng/ml, with the accuracy 106.7% and RSD 10.1%. According to Chinese Pharmacopoeia, the LOQ could be validated when the accuracy is in the range of 80–120% and RSD is lower than 20%. Therefore, the LOQs for rivanol and mifepristone of this assay estimated according to the signal-to-noise ratio were reliable.

3.5. Precision and accuracy

Validation of the proposed method was carried out using comparisons of inter-day and intra-day variation and accuracy of the three QC samples at the concentration of 10, 100 and 1000 ng/ml. Inter-day precision and accuracy were evaluated in one working day in six replicates of QC samples at low, medium and high concentrations of rivanol and mifepristone in the plasma matrix, while intra-day precision and accuracy were evaluated in six replicates in three working days (Table 2). Precision was presented as the coefficient of variation (CV), and accuracy was expressed as percentage recovery of QC samples. For rivanol, the inter-day CV values were less than 6.7%, while the intra-day CV values were less than 7.8%. And the accuracy was within 95.3–103.9% when compared with nominal concentrations. For mifepristone, the inter-day CV values were less than 9.3%, while the intra-day CV values were less than 10.1%. And the accu-

Table 2
Inter- and intra-day precision and accuracy for rivanol, mifepristone and IS in spiked plasma

Compound	Concentration added (ng/ml)	Inter-day			Intra-day		
		Concentration found (ng/ml)	CV (%)	Mean accuracy (%)	Concentration found (ng/ml)	CV (%)	Mean accuracy (%)
Rivanol	10	9.53 ± 0.64	6.7	95.3	9.61 ± 0. 75	7.8	96.1
	100	97.6 ± 5.1	5.2	97.6	103.9 ± 6.4	6.2	103.9
	1000	999 ± 43	4.3	99.9	1025 ± 47	4.6	102.5
Mifepristone	10	9.38 ± 0.87	9.3	93.8	9.39 ± 0.95	10.1	93.9
	100	96.5 ± 5.4	5.6	96.5	97.2 ± 6.3	6.5	97.2
	1000	988 ± 52	5.3	98.8	1013 ± 54	5.3	101.3
IS	1000	1023 ± 26	2.5	102.3	1041 ± 49	4.7	104.1

racy was within 93.8–101.3% when compared with nominal concentrations. The results indicate that the method is reliable, reproducible, and accurate for both rivanol and mifepristone.

3.6. Stability

As rivanol is sensitive to light, samples should be stored lightproof properly. The long term stability of plasma samples was evaluated by the difference in concentration found after being stored for 15 days at −20 °C, and expressed by the relative variation (%) which was calculated as [(concentration found − concentration added) × 100/concentration added]. Three QC samples at concentrations of 10, 100 and 1000 ng/ml were used to evaluate it, and then the average relative variations ($n = 6$) of rivanol were −4.2%, −3.8% and −3.1%, respectively. And, those of mifepristone were −2.9%, −3.5% and −1.7%, respectively. Results of the stability experiments indicated that plasma samples were stable for at least 15 days when stored lightproof at −20 °C. To evaluate autosampler stability, the three QC samples pretreated as mentioned above were tested in triplicate at three different times, immediately after preparation, and after 24 h maintained lightproof at 4 °C, the anticipated time the batch size would take to run, and after 36 h, the anticipated testing time. The stability of the analytes and the internal standard was checked by the mean recoveries against the same samples injected immediately after preparation. The mean recoveries were all higher than 95%, suggesting the autosampler stability was good.

3.7. Method application

The developed assay was subsequently applied to the determination of plasma samples from the five volunteered women.

Table 3
Plasma levels of rivanol and mifepristone of the five volunteered women

Code	Rivanol concentration (ng/ml)	Mifepristone concentration (ng/ml)
1	19.6	291
2	9.3	76
3	8.4	126
4	22.5	154
5	25.7	328

Results are shown in Table 3, which were reported first time for polypharmacy with rivanol and mifepristone. The detected levels of both drugs vary much in these women, perhaps due to various factors such as the metabolic characteristics of the two drugs, the intra-individual variability in absorption, metabolism and elimination of the two drugs, and the weight, and so on.

4. Conclusion

A HPLC-UV method is developed for the simultaneous determination of rivanol and mifepristone in human plasma. Baseline separation of the two analytes and the IS was achieved in less than 15 min. The analytes were preconcentrated by the aid of SPE, and good extraction efficiencies were obtained. Satisfactory precision and accuracy were demonstrated. This method has been successfully applied to assess simultaneously the plasma level of rivanol and mifepristone in young women undergoing polypharmacy with the two drugs.

Acknowledgements

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