Determination of ng Rivanol in Human Plasma by SPE-HPLC Method

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Abstract

A high-performance liquid chromatography assay is described for the determination of rivanol in human plasma. Solid-phase extraction cartridges are used to extract plasma samples. Separation is done by using a C_{18} column. The mobile phase is a mixture of methanol–0.05% sodium dodecylsulfonate (70:30, v/v, pH 3), with the flow rate at 1.0 mL/min. UV detection of rivanol is at 272 nm. The calibration curve is linear in the concentration range of 1×10^{-8} mol/L to 1×10^{-5} mol/L with linear correlation coefficient *r* equal to 0.9998. The limit of detection for the assay is 3×10^{-9} mol/L, corresponding to 1.1 ng/mL. Precision, expressed as the within- and between-day coefficient of variation, is 3.3–8.1% and 4.1–9.5%, respectively, at plasma control samples of 5×10^{-8}, 5×10^{-7}, and 5×10^{-6} mol/L. And the recovery ranges from 94.8% to 107.2%. The selectivity of the method is confirmed. Plasma samples are stable for at least 15 days if they are stored lightproof at –20°C. This method is simple, sensitive, and accurate, and it allows for the determination ng rivanol in human plasma. It could be applied to assessing its plasma level in women receiving an intra-amniotic injection of rivanol.

Introduction

Rivanol, also known as ethacridine lactate, is 6,9-diamino-2-ethoxyacridine lactate, and its structure is given in Figure 1. It was employed as a potent antimicrobian agent from the pre-penicillin era and in various other tests on antigens too. In addition, it was a drug commonly used for second trimester termination of pregnancy, which has proved to be associated with the lowest rate of complications (1). Transcervical extraamniotic route for the application of rivanol is often chosen, because of its similar effect and fewer side effects in comparison with modern vaginal prostaglandins (2,3). Nevertheless, a technique that injects rivanol into the amniotic cavity to terminate the second trimester pregnancy has been experimented in China since the 1980s (4), and this could avoid the shortcomings brought by extraamniotic administration, such as the need for cervical manipulation, the risk of systemic absorption of rivanol, the risk of infection, and so on. From then on, this method has drawn a lot of attention from researchers in the Western world too (5,6). Now in China, intraamniotic administration of rivanol is widely and legally used as an abortion route in government agencies or public hospitals concerned. However, due to the family planning project in China, rivanol is abused illegally in recent years by some people to induce termination of the second trimester pregnancy and expulsion of a female fetus examined by B ultrasound, in order to acquire a second pregnancy opportunity for a male fetus. This kind of gender-select behavior has led to a serious unbalance of the sex ratio at birth in China, whose value was recently reported by the government to be approximately 120:100 and even higher than 200:100 in some remote territories (7). To help prevent an increase in this imbalance, it has become important to develop a sensitive and validated method for the qualitative and quantitative determination of rivanol in biological samples, thus proving the illegal abuse of rivanol.

Several methods have been used to analyze rivanol, including titration with tetraphenylboron (8) or bismuth nitrate (9), fluorescence spectra (10,11), catalytic photokinetic method (12), spectrophotometric analysis (13), electroanalytical method (14,15), etc. Obviously, the qualitative and quantitative abilities of those methods were not so good as to determine ng rivanol in biological samples. A high-performance liquid chromatography (HPLC) method was also used in several references. The one described by Wu (16) used an Alltech C8 (250 × 4.6 mm, 5 µm) column as the analytical one and 0.05% sodium laurysulfonate–methanol (30:70, v/v) as the mobile phase. Another

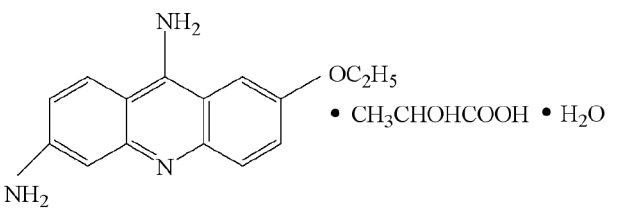

Figure 1. Chemical structure of rivanol.

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method developed by Mi et al. (17) used an octadecylsilyl silica (ODS) (150 × 4 mm, 10 µm) column and methanol–acetonitrile (ACN)–0.1 mol/L solution of ammonium acetatc (55:10:35) as the mobile phase. The third one developed by Yoshinobu et al. (18) achieved the separation employing a 25-cm column packed with Zorbax ODS, using 5mM Na lauryl sulfate in 95% ACN as an eluent. However, the previously mentioned methods used tablets instead of biological samples as the matrix for analysis. The HPLC method developed by Zheng et al. (19) could be used to determine rivanol in human urine, in which urine samples were extracted with aether and separated by a µ-Bondapak C18 column (300 × 3.8 mm) using ethanol–water (80:20, v/v) as the mobile phase. However, this method was not validated. Besides, because it was a liquid–liquid extraction procedure, the concentration range of the calibration curve was very narrow (2.5–20 ng/mL), and plasma samples were not experimented. The object of this study was to develop a simple, sensitive, and validated HPLC method to determine ng rivanol in human plasma samples.

Experimental

Materials and reagents

Rivanol was purchased from Sigma-Aldrich (St. Louis, MO), with the purity reported as 99%. HPLC-grade methanol and ACN were purchased from Tedia Company (Fairfield, OH). Ultrapure water (18 MOhms) was achieved by distilling twice and deionizing by ion-exchange resin, and then reversing the osmosis membrane in a HB-RO/10 deionzation ultrapure system (Huibang, Hangzhou, Zhejiang, China). All other chemicals and solvents were analytical reagents and obtained from common commercial sources. Extraction cartridges (Oasis MCX 1 mL, 30 mg) were purchased from Waters (Milford, MA).

Instrumentation and operating parameters

HPLC determinations were performed with a Shimadzu model 10ATvp LC system (Chiyoda-Ku, Tokyo, Japan), consisting of two LC-10AT$_{VP}$ pumps, a DGU-20A3 on-line degasser, an SPD-10A$_{VP}$ UV detector, a CTO-10A column oven, and a Rheodyne 7725i manual injector (Cotati, CA) 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 was provided by the N2000 computer software package (Zhida, Hangzhou, Zhejiang, China). The separation was performed on a Shimadzu Shim-Pack VP-ODS reversed-phase column (150 × 4.6-mm i.d., 4.6-µm particle size) protected by a Shimadzu Shim-Pack guard column (10 × 4.6-mm i.d., 4.6-µm particle size). The mobile phase was a mixture of methanol–0.05% sodium dodecylsulfonate (70:30, v/v), using concentrated H_3PO_4 to adjust the pH of the mixed solution to 3. The flow rate was set at 1.0 mL/min. The mobile phase was prepared fresh daily and filtered using a 0.2-µm poly(vinyldene fluoride) (PVDF) filter prior to use. The column effluent was monitored at 272 nm with UV detection. The column temperature was regulated at 28°C. Data were collected and integrated by using N2000 software and analyzed by using Microsoft Excel 97.

Preparation of standards and controls

A concentrated stock solution of rivanol was prepared at a concentration of 1×10^{-3} mol/L in ultrapure water and was further diluted with water into 1×10^{-7} mol/L – 1×10^{-4} mol/L for the preparation of plasma calibration standards. All solutions were stored at –20°C in cleaned brown glass wrapped by black paper. Different standard solutions (100 µL each) were transferred into tubes containing 900 µL of drug-free human plasma to obtain plasma calibration standards at the concentration range of 1×10^{-8} mol/L – 1×10^{-5} mol/L. Quality control (QC) samples were also prepared as introduced at the concentration of 5×10^{-8}, 5×10^{-7}, and 5×10^{-6} mol/L. These calibration standards and QC samples were then treated following the same procedure as follows.

QC samples to determine accuracy and precision of the method were independently prepared at low (5×10^{-8} mol/L), medium (5×10^{-7} mol/L), and high (5×10^{-6} mol/L) concentrations in the same manner as the calibration standards and stored at –20°C before use.

Sample collection

Sample collection was carried out in the Affiliated Hospital of Anhui Research Institute for Family Planning located at Hefei in China. Five healthy women, 22–28 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, they volunteered to abort by using rivanol in the government hospital and they also volunteered to join in this study. All were of normal weight, with the body mass index varying from 19.0 to 24.5 kg/m^2. Prior to participation, each woman signed an informed consent document.

Each volunteer received a single intra-amniotic injection of 100 mg rivanol at 0 h. When the abortion was accomplished, with the time ranging from 38.5 to 47.2 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.

Sample preparation and extraction procedure

Plasma samples were thawed at room temperature and vortexed for 5 min prior to use. All samples, including blanks, standards, QCs, and unknowns, were extracted using the previously mentioned solid-phase extraction (SPE) cartridges. Each cartridge was activated by 1 mL methanol and then balanced by 1 mL water to prepare sorbent for use. Though it was claimed that this kind of cartridge afforded to extract 1 mL sample, plasma samples would often plug up the cartridge in some cases. Thus, the volume of samples was selected as 0.5 mL instead of 1 mL to avoid this. Plasma samples were acidified by adding 20 µL concentrated H_3PO_4. Thus, rivanol, a basic drug, was in the ionized form for retention by cation exchange, and the drug–protein interaction could be disrupted effectively at a low pH, simultaneously. Then plasma samples

were loaded to pass through the cartridges, and the cartridges were washed through three sequential steps. First, 1 mL 0.1 mol/L HCl was added to remove proteins and lock rivanol to sorbent by ion-exchange mechanism. Second, 1 mL 100% methanol was used to remove interferences retained by hydrophobic interaction. And finally, 1 mL 1 mol/L NaOH was added to counteract the superfluous acid and unlock rivanol from sorbent. At last, the analytes were eluted with 1 mL of acetonitrile–20% ammonium hydroxide (80:20, v/v) and collected in clean glass tubes. All previously mentioned steps were carried out without lab vacuum. Occasionally, a 1-mL injector was plugged into the end of the cartridges to force the solutions to pass through it in 5 min. The eluent was evaporated to dryness under –0.08 MPa lab vacuum at 50°C, and the extraction residue was reconstituted in 50 µL of 100% methanol just prior to injection into the HPLC system. Then, all of the achieved aliquot was manually injected to insure that the 20-µL loop of the injector was totally full.

Attentively, as the aqueous solution of rivanol is photosensitive, the lightproof measure should be implemented throughout the process. Our experiments showed that approximately 5% of rivanol at the concentration of 1×10^{-4} mol/L would be photodegraded in an hour when exposed to sunlight. Thus, most of the operation, including preparation of solutions, sample collection, sample preparation and pretreation, and chromatographing, was carried out in a dark room. All solutions were stored in cleaned brown glass wrapped in black paper or cloth.

Results and Discussion

Method development

If a mixed solution of methanol–water (70:30, v/v) was used as the mobile phase, the chromatographic peak of rivanol would tail severely, with the tailing factor higher than 10. It was perhaps because of the secondary retention effect caused by the interaction between the polar groups in rivanol and the residual silanol on the bonded stationary phase. When 0.05% sodium dodecylsulfonate solution at pH 3 instead of pure water was used, rivanol changed to the ionized form in acidic environment and combined with the dodecylsulfonate group, acting as the counter-ion, to form an ion-pair that exhibited very good retention characteristic on the C_{18} column. The chromatographic peak of rivanol was then very symmetrical, with the tailing factor no more than 1.1 at 10% of peak height.

As advised by the manufacturer, 1 mL 5% NH_4OH in methanol could be used to elute the basic drug of interest for the Oasis MCX cartridges. However, in this case, rivanol could not be eluted, and even the elution was adjusted to 1 mL of acetonitrile–20% NH_4OH (80:20, v/v). After some experiments, it was found that if 1 mL of 1 mol/L NaOH was used to wash the cartridge before the elution with 1 mL of acetonitrile-20% NH_4OH (80:20, v/v), rivanol could then effectively be eluted and the recovery was satisfying. The reason was perhaps that the alkalinity of NH_4OH was not strong enough to break the interaction between rivanol and the strong cation-exchange sulfonic acid groups on the surface of sorbent in the MCX cartridge, though NaOH could.

Selectivity and chromatography

The degree of interference by endogeneous plasma constituents with rivanol was assessed by inspection of chromatograms derived from a processed blank plasma sample. A series of typical chromatograms obtained from standard solution, blank human plasma, blank human plasma spiked with rivanol, and a human plasma sample collected from a volunteer were presented (Figure 2). Elution time of rivanol was 7.46 ± 0.06 min ($n > 20$). A good separation of rivanol was obtained under the chromatographic conditions described previously. Although there were characteristic peaks for the biological matrix before 7 min, there were not any interfering peaks after 7 min, depicting no interfering peaks at the corresponding elution time of rivanol. The peak width was a little bigger in Figure 2D than in Figure 2A because of the degeneration of the column, which had a minimum of 200 injections of biological samples between them.

Linearity and sensitivity

The calibration curve for rivanol was constructed by plotting peak area y (µV·s) versus rivanol concentration in plasma x (mol/L) over five independent runs with concentrations of 0, 1×10^{-8}, 5×10^{-8}, 1×10^{-7}, 5×10^{-7}, 1×10^{-6}, 5×10^{-6}, and 1×10^{-5} mol/L. It was linear with a correlation coefficient of 0.9998 ± 0.0001 over the range, with a slope of $(9.428 \pm 0.853) \times 10^{10}$, and

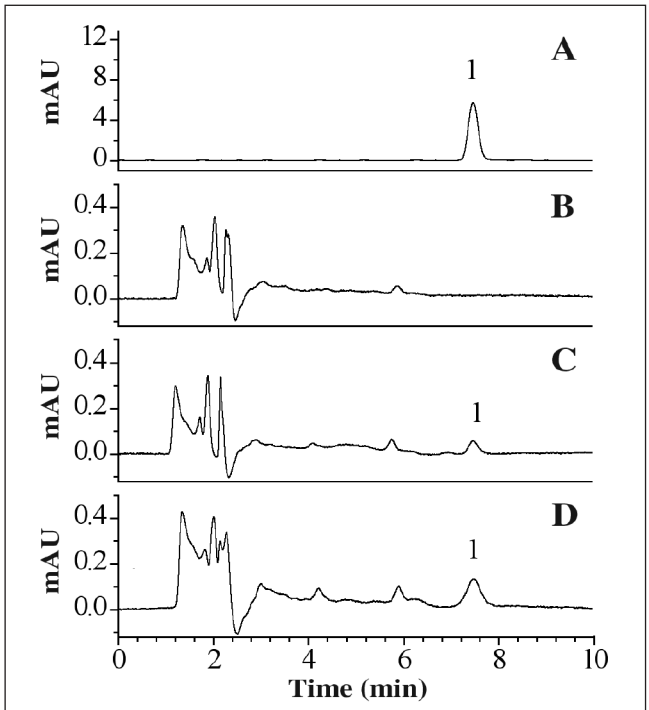

Figure 2. HPLC chromatograms of rivanol (1) in: standard solution spiked with 1×10^{-5} mol/L of rivanol injected into the HPLC system directly (A); blank human plasma (B); human plasma spiked with 1×10^{-8} mol/L of rivanol (C); and a human plasma sample collected when the abortion was accomplished after the intra-amniotic injection of 100 mg rivanol (3.24×10^{-8} mol/L) (D).

Table I. Within- and Between-Day Precision and Accuracy for Determination of Rivanol in Spiked Plasma

	Within-day			Between-day		
Concentration added (mol/L)	Concentration found* (mol/L)	CV (%)	Relative error (%)	Concentration found* (mol/L)	CV (%)	Relative error (%)
5×10^{-8}	$5.416\ (0.438) \times 10^{-8}$	8.1	8.3	$5.541\ (0.528) \times 10^{-8}$	9.5	10.8
5×10^{-7}	$5.112\ (0.167) \times 10^{-7}$	3.3	2.2	$5.147\ (0.211) \times 10^{-7}$	4.1	2.9
5×10^{-6}	$4.878\ (0.243) \times 10^{-6}$	5.0	–2.4	$4.816\ (0.296) \times 10^{-6}$	6.1	–3.7

* Mean (standard deviation), $n = 6$.

an intercept of $(1.576 \pm 0.569) \times 10^3$. The limit of detection (LOD) for the assay of rivanol was calculated based on a signal-to-noise ratio of 3:1, which was approximately 3×10^{-9} mol/L, corresponding to 1.1 ng/mL. And the limit of quantitation (LOQ), based on a signal-to-noise ratio of 10:1, was approximately 1×10^{-8} mol/L, corresponding to 3.6 ng/mL (20).

Precision and accuracy

Within-day precision and accuracy were evaluated by one working day in six replicates of quality control samples at three different concentrations of rivanol, and between-day precision and accuracy in six replicates were evaluated in three working days (Table I). Precision was presented as the coefficient of variation (CV), and accuracy was expressed as a relative error [(concentration found – concentration added)/concentration added] × 100(%)]. Within- and between-day relative standard deviations (RSDs) were less than 8.1% and 9.5%, and accuracies were within 8.3% and 10.8%, respectively. The results indicate that this method is reliable, reproducible, and accurate.

Recovery and stability

Recovery was calculated by comparing the peak areas obtained from the QC samples with those from the aqueous solutions containing the same amount of rivanol. The mean recoveries of rivanol from plasma performed at three representative concentrations of 5×10^{-8}, 5×10^{-7}, and 5×10^{-6} mol/L, expressed as mean ± RSD, were 107.2 ± 8.4%, 94.8 ± 2.5%, and 96.3 ± 2.6%, respectively.

Because of the high sensitivity of rivanol to light, all types of rivanol samples should be properly stored in lightproof containers. The stability of rivanol in human plasma was evaluated by the difference from the concentration found after being stored for 15 days at –20°C, and it was expressed by the relative variation (%), which was calculated as [(concentration found – concentration added) · 100/concentration added]. The average relative variations ($n = 6$) of three QC samples at concentrations of 5×10^{-8}, 5×10^{-7}, and 5×10^{-6} mol/L were –4.8%, –2.7%, and –2.5%, respectively. Results of the stability experiments indicated that rivanol in the plasma samples was stable for at least 15 days when stored in lightproof containers at –20°C.

Method application

Human plasma samples were collected as previously described and analyzed using this method. Expressed by mean (standard deviation) ($n = 6$), the concentrations were 3.24 $(0.29) \times 10^{-8}$, 6.89 $(0.72) \times 10^{-9}$, 1.15 $(0.25) \times 10^{-8}$, 4.83 $(0.30) \times 10^{-8}$, and 2.77 $(0.21) \times 10^{-8}$ mol/L, corresponding to 11.74 (1.05), 2.50 (0.26), 4.17 (0.91), 17.50 (1.09), and 10.04 (0.76) ng/mL, respectively. The described results were approximately consistent with previous data reported by Wu et al. (11), and 3 ~ 8 ng/mL was determined by a fluospectrophotometric method.

Conclusion

An SPE-HPLC method for the determination of ng rivanol in human plasma was developed and validated. This method showed good reproducibility and accuracy with the calibration curve ranges of 1×10^{-8} to 1×10^{-5} mol/L, and it had a low LOD of 3×10^{-9} mol/L, corresponding to approximately 1.1 ng/mL. It was successfully applied to assessing the level of rivanol in human plasma for those women at childbearing age.

Acknowledgments

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