Determination of rosmarinic acid and its N-substituted analog A1 in rat plasma by high-performance liquid chromatography-tandem mass spectrometry and its application in pharmacokinetics

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Abstract

The researcher will introduce the way of effective liquid chromatography tandem mass spectrometric method (HPLC-MS) which is quick, sensitive, and alternative to determine the (E)-3-(3,4-dihydroxyphenyl) -2-(3-(3,4-dihydroxyphenyl)acrylamido)propanoic acid (A1) and rosmarinic acid (RA) of the plasma in rats. The analyses were divided into a C18 column (1.9 μm, 2.1 mm × 100 mm) with a security guard C18 column (5 μm, 2.1 mm × 10 mm) and a triple-quadrupole mass spectrometry with an electrospray ionization (ESI) ion-source generates ions. With Pseudoephedrine hydrochloride being a standard, the sample pretreatment is relevant to the one-step protein precipitation with isopropanol: ethyl acetate (v/v, 20:80). This method presented a linear relationship within ranges of the concentration of 5–750 ng/ml for RA and A1. Relative standard deviations (RSD) in daily courses stood less than 15% and the relative errors (RE) registered within 15%. The means adopted in this research makes the RA's and A1's unambiguous quantification and identification and in vivo possible. And this study can be the first one to focus on determining the A1 and RA of rat plasma with administration orally. The results served as a significant basis to evaluate the medicine's applications in the clinic.

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Introduction

Caffeic acid esters, rosmarinic acid (RA, Fig. 1a), exhibit different biological properties, such as antiviral, antibacterial, anti-inflammatory, and antioxidant properties[1,2]. It has been recently noted that RA pharmacology is important[2–5], but very few papers have evaluated its pharmacokinetics and pharmacodynamics [6–11]using high-performance liquid chromatography-tandem mass spectrometry. In order to investigate the pharmacodynamic mechanisms of RA in more depth, we synthesized many analogs based on RA, including (E)-3-(3,4-dihydroxyphenyl)-2-(3-(3,4-dihydroxyphenyl)acrylamido)propanoic acid (A1, Fig. 1b). Previous studies have shown that A1 has higher pharmacological activity against tumors than RA[12]. Thus, in order

to simultaneously determine A1 and RA, pharmacokinetics (PK) must be studied using a sensitive and precise method. Recently, researchers have expressed concern about RA absorption and metabolism, but few papers have used HPLC-MS to study the pharmacokinetics and pharmacodynamics of RA. By avoiding the need to fully analyze the chromatograms, this method guarantees high sensitivity and speedy quantification. The majority of previous studies, however, only focused on administering one substance at a time; thus, no one has conducted a simultaneous study on RA and other compounds and their pharmacokinetics. The objective of this study was to implement and validate a new method for estimating RA and A1 in plasma samples collected from rats. Therefore, this method is very useful for pharmacokinetics studies.

2. Experimental section

2.1. Reagents and chemicals

RA (batch number 111871) with a purity above 99% and pseudoephedrine hydrochloride (PPD, batch number 171237-201510) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) (Fig. 1c) and used as standards. A1 (99.8% purity) was synthesized at the School of Pharmaceutical, Guangxi Medical University (Nanning, China). Ethyl acetate was obtained from Dikma Technologies, Inc. (Richmond Hill, ON, Canada). The methanol and acetonitrile used were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Deionized water was purified with an Alpha-Q water purification system and then filtered with 0.20-μm membranes.

2.2. Instruments and conditions

A Dionex UltiMate 3000 HPLC system with a binary pump, an on-line degasser, etc. was employed for analysis. Separation was carried out by chromatography at 40 °C using a Hypersil GOLD C18 column (100 mm x 2.1 mm, 1.9 mm particle size) and C18 guard column. Acetonitrile-0.1% formic acid (25:75, v/v) was introduced at a flow rate of 0.2 ml/min. Moreover, 2 μl aliquots were added to the HPLC system for analysis.

In order to analyze MS data, a Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole mass spectrometer was operated in negative ionization mode with an electrospray ionization source (ESI).The typical parameters of the ion source were as follows: sheath gas pressure (N_2): 20 units; spray voltage: 3500 V; collision gas pressure (Ar): 1.5 mTorr; and scan width: 0.002; selected-reaction monitoring (SRM) with monitoring ion pairs was used to analyze the samples at m/z 359 - 161 for RA, 358 - 161 for A1, and 164 - 146 for the internal standard (IS). Every channel had a scanning dwell time of 0.1 s. Xcalibur 2.2 software was used in the centroid mode to acquire and process data.

2.3. Preparation of standards and quality control samples

By dissolving 10 mg of A1 or RA in 100 ml of methanol, the standard stock solution was prepared. Then, individual solutions were diluted with methanol to achieve ideal concentrations and used as standard solutions. We also dissolved and diluted PPD (10.0 mg) in methanol to obtain a stock solution with a concentration of 1.0 mg/ml, which was subsequently diluted to obtain an IS working solution at a concentration of 5.0 μg/ml. These solutions were kept at 4 °C and subsequently exposed to normal temperature prior to usage. One hundred microliters of blank plasma was spiked with 50 μl of appropriate RA and A1 standard solutions to obtain RA or A1 concentrations of 5, 25, 50, 100, 250, 500 and 750 ng/ml, and calibration standards were used every day. In a similar manner, quality control (QC) samples were prepared at low, medium, and high concentrations to obtain samples containing 10, 100, and 500 ng/ml RA and A1, respectively.

2.4. Plasma sample preparation

The mixtures were put into 1.5-ml microcentrifuge tubes containing 20 ml of standard solution and 100 ml of plasma. Then, 1000 μl of isopropanol:ethyl acetate (20:80, v/v) was injected into the tubes and vortexed for 60 s. The tubes were subsequently centrifuged at 8049.6×g for 10 min. Another tube was used for the organic layer, which was subsequently evaporated to dryness. After dissolving the residue in 100 μl of mobile phase, 2 μl of the extract was injected into the HPLC-MS/MS system. SYKG2003-0005 is the license number of the Animal Protection and Utilization Committee at Guangxi Medical University that approved this study.

2.5. Method validation

We evaluated the selectivity by comparing the chromatograms of six batches of blank rat plasma with spiked rat plasma. The linearity of six calibration curves was determined through a weighted ($1/x^2$) analysis. By analyzing three samples (n = 6) of low-, medium-, and high-concentration QCs on different days, we determined intraday and interday precisions (relative standard deviation, RSD) and accuracy(relative error, re). The matrix effect was studied by comparing the peak areas of the analytes in low-concentration and high-concentration postextraction spiked blank plasma samples with those of the corresponding standard solutions. In order to determine the recovery rate, six extracted samples were compared to six extracted samples with low, medium, and high QC concentrations. Stability was evaluated by comparing the repeatability of low and high QC samples (n = 6) during storage and processing. A freeze-thaw stability test was performed after three freeze-thaw cycles. By analyzing the QC samples at 4 °C in one day, the stability of the samples during postpreparation was assessed. For long-term and short-term stability evaluations, six aliquots of QC samples were stored at 20 °C for two weeks and at ambient temperature for four hours.

2.6. Pharmacokinetic (PK) study in rats

Male Sprague–Dawley rats weighing 250-300 g were selected for the PK study. All animal experiments were conducted according to the guidelines of the institutions and with the approval of the Use and Care of Animals of Guangxi Medical University. Twelve rats were individually treated with RA and A1 aqueous solutions at 10 mg/kg. We collected blood samples (0.5 ml) at 0, 5, 15, 30, 45, 60, 90, 120, 240, 360, 480, and 720 min following oral administration of the drug. These samples were stored in heparinized tubes. After centrifugation at 8049.6×g and 4 °C for 10 min, plasma was collected and frozen at -20 degC until analysis. Using TopFit 2.0 software, pharmacokinetic parameters were assessed in a noncompartmental model. The elimination half-life ($t_{1/2}$) is 0.693 k_e, where k_e is the elimination rate constant, which is calculated by fitting the mean data of the plasma concentration curve at 4 end points with a logarithmic linear regression equation using the least squares method. Based on the observed data, we calculated the maximum drug plasma concentration (Cmax) and time to reach Cmax (Tmax). The area under the plasma concentration-time curve from zero to the final measurable sample time (AUC_{0-t}) was calculated using the linear trapezoidal rule.

3. Results and discussion

3.1. Chromatography and mass spectrometry

We optimized the chromatographic conditions by using various proportions of methanol in water to achieve optimal peak separation and peak shape with little peak tailing in a short analysis time, but very poor chromatography was achieved. Acetonitrile and 0.1% formic acid in water (25:75, v/v) were employed. The final chromatographic conditions showed that PPD behaved similarly to RA in terms of retention and ionization, and was adopted as the IS.

Methanol was first tested as a pretreatment method for protein precipitation. However, methanol caused poor efficiency in extracting A1 in this study. Hence, we introduced isopropanol:ethyl acetate (50:50, v/v) to increase the efficiency of sample extraction, which provided cleaner extracts and higher IS recoveries. By adjusting the ratio of isopropanol and ethyl acetate (20:80, v/v) to extracts, the recovery rates were further increased without compromising extract cleanliness.

A more accurate response for the two analytes was achieved using the ESI source when compared with the response using the APCI source. In full scan precursor ion spectra, the most abundant ions of A1, RA, and IS should be deprotonated molecules with m/z 358, 359, and 164, respectively (Fig. 2). Parameters such as the ESI source temperature and flow rate of the desolvation gas have been enhanced to obtain the maximum response of deprotonated molecules. To obtain the maximum response of the compound fragments, collision-induced decomposition (CID) and the SRM collision gas pressure and energy were selected through the precursor - product ion transition. The product ion spectra revealed abundant ions in fragments at m/z 161 for RA and A1 and 146 for the IS. The characterization of the analyte fragments is shown in Fig. 3.

3.2. Method validation

3.2.1. Specificity

RA, A1 and IS chromatograms obtained from processed blank plasma samples were examined to determine the degree of interference by endogenous plasma components. Fig. 4(a)–(c) shows typical chromatograms of blank plasma, plasma spiked with IS and plasma samples with A1 and RA. The retention times of PPD (IS), RA, and A1 were 1.15, 1.38, and 1.53 minutes, respectively, with no endogenous interference or matrix effects.

3.2.2. Linearity and LLOQ

RA and A1 calibration curves showed good linearity over the concentration range of 5-750 ng/ml. The typical calibration plot equations and their correlation coefficients were calculated as follows: RA: $y = 2.01$ x 10^{-3} $x + 1.12$ x 10^{-1} ($r^2 = 0.9987$); A1:$y = 2.99$ x 10^{-4} $x + 3.80$ x 10^{-3} ($r^2 = 0.9995$). In the equation $y = ax + b$, x is the concentration of the analyte in the serum, and y is the peak area ratio of the analytes to the IS. The lower limit of quantitation (LLOQ) of RA and A1 is 5 ng/ml, and the coefficients are 8.14% and 7.32%, respectively. The signal-to-noise ratios of RA and A1 (SN) are 97 and 102, respectively.

3.2.3. Precision and accuracy

The intraday accuracy, interday accuracy and precision were obtained by measuring the QC samples at 3 concentration levels, as described in Section 2. The intraday accuracy of RA detection was -0.21-1.32%, with precision below 2.39%, and that of A1 detection was -1.03-2.01%, with precision below 3.51%. The interday accuracy of RA detection was -2.53-0.42%, with precision below 1.32%, and that of A1 detection was -0.91-3.01%, with precision below 4.05%. Table 1 shows that the method's overall reproducibility was acceptable.

3.2.4. Extraction recovery and ionization

The mean extraction recoveries obtained for 6 replicates of the QC samples at different concentrations were 89.52 +- 4.35% (precision, 9.2%), 87.23 +- 5.17% (8.2%), and 90.41 +- 3.65% (8.2%) for RA, 85.46 +- 4.19% (6.60%), 88.23 +- 4.37% (7.60%), and 84.98 +- 5.14% (7.60%) for A1 and 92.18 +- 6.16% (6.60%) for the IS. These data were calculated with precision.

In terms of ionization, the peak area ratios of the two target compounds and IS after spiking the evaporated plasma samples at three concentration levels were 96.8-103.2% and 95.3-103.6% of the concentration levels of the neat standard solutions of RA and A1, respectively, which suggests that the method was free from matrix effects.

3.2.5. Stability

As shown in Table 2, RA and A1 stabilities in the processed samples have been assessed after freeze-thaw cycles and long-term cold storage at -20 degC for 14 days. The results show that the three analytes can be kept stable for 24 h under the conditions of the autosampler after preparation, 14 days under refrigeration, and through 3 freeze-thaw cycles in plasma samples. Since the concentrations of RA and A1 have not significantly changed, the RA and A1 in plasma should be tested within a specified time under specified storage conditions.

3.3. Pharmacokinetic studies

With oral administration of 10 mg/kg RA and A1 to 12 individual Sprague–Dawley rats, this method was validated and proven to be suitable for use in PK studies. The concentration-time data of A1 and RA corresponded to the two-compartment pharmacokinetic model. Fig. 5 shows the mean plasma concentration-time curve (mean +- SD) of RA and A1 after administration of a dose. In spite of dosage differences, these RA values did not differ significantly from those reported by Yang et al.[13] and Noguchi-Shinohara et al.[14]. By replacing the O atom with an N atom, no significant difference was observed between the times to reach the maximum A1 and RA plasma concentrations; however, C_{max} increased from 209.3 +- 9.4 to 255.0 +- 20.8

min, and the area under the plasma concentration-time curve from 0 min to the time of the last measurable concentration (AUC_{0-t}) was twice that of RA, 16,859.2 +- 1,174.1 ng/ml min, amounting to 36,677.5 +- 4,493.6 ng/ml min. At the terminal phase, the drug elimination half-life ($t_{1/2}$) also increased from 108.4 +- 13.1 to 366.3 +- 12.2 min. Obviously, from the results, A1 has a higher oral bioavailability than RA, which may indicate a direction for the structural modification of such substances. The mechanisms of A1 need to be clarified by additional studies.

4. Discussion

The current research hotspot is to modify the structure of RA to obtain drugs with superior efficacy. We prepared N-substituted RA compounds and completed a preliminary pharmacodynamic evaluation. To clarify the mechanisms of A1, pharmacokinetic studies of A1 and RA are required. In this study, a sensitive, simple and rapid HPLC–MS/MS method was adopted to simultaneously analyze A1 and RA in a single-dose study on rat plasma. It has been successfully applied to the PK study of RA and A1 in rats. This result may provide a reference for the modification of the RA structure.

Author Contributions

Conceived and designed the experiments: SF, WZ and MJ. Performed the experiments: MJ, SF, QZ and SZ. Analyzed the data: MJ. Contributed reagents/materials/analysis tools: MJ, SF and QZ. Wrote the paper: MJ.

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Fig. 1. Chemical structures of RA (A), A1 (B) and PPD (C).

Fig. 2. Precursor ion and product ion spectra of RA (a), A1 (b) and PPD (c).

Fig. 3. Characterization of the analyte fragments. (a) m/z 359 - 161 for RA; (b) m/z 358 - 161 for A1; and (c) m/z 164 - 146 for PPD.

Fig. 4. Representative SRM chromatograms of PPD (IS, I), RA (II) and A1 (III) in rat plasma: (a) a blank rat plasma sample; (b) a blank rat plasma sample spiked with PPD (100 ng/ml), RA (10 ng/ml), and A1 (10 ng/ml); (C) a rat plasma sample 30 min after an oral dose of RA and A1 was administered at 10 mg/kg (calculated as RA) to a Sprague–Dawley rat.

Fig. 5. Mean plasma concentration–time profiles of RA and A1 administered to 12 Sprague–Dawley rats (mean +- SD).

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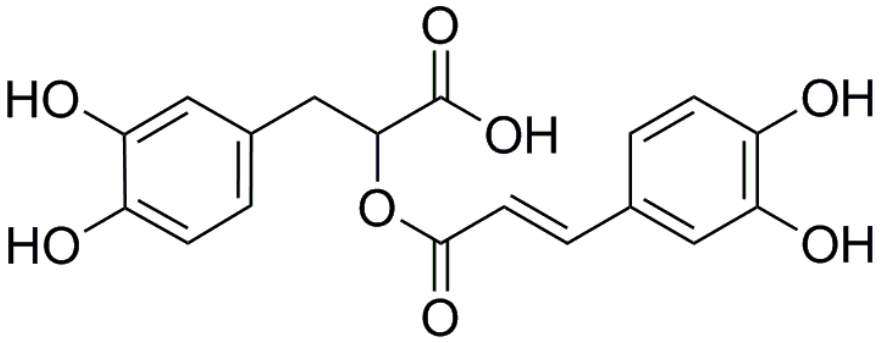

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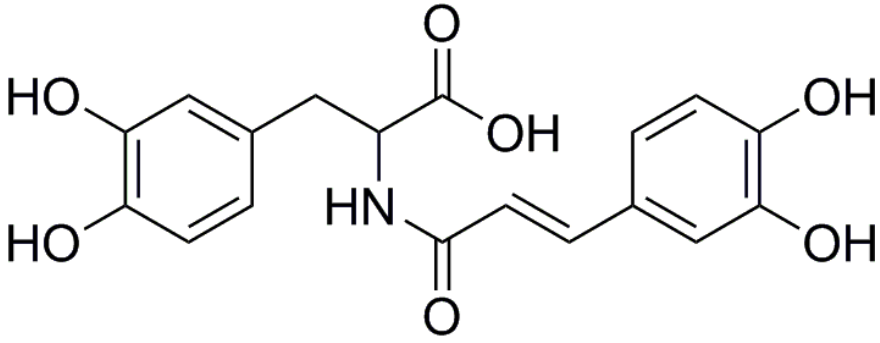

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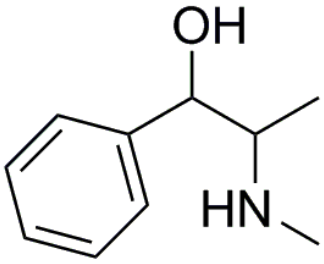

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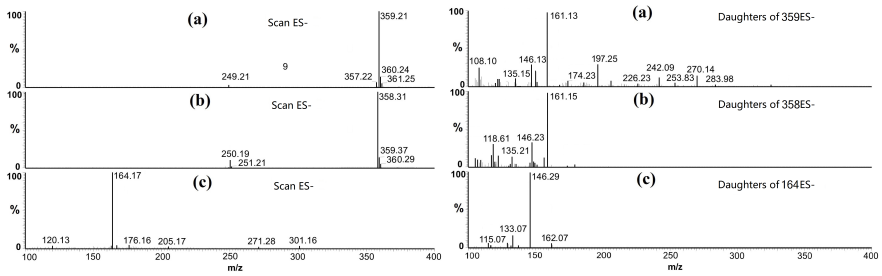
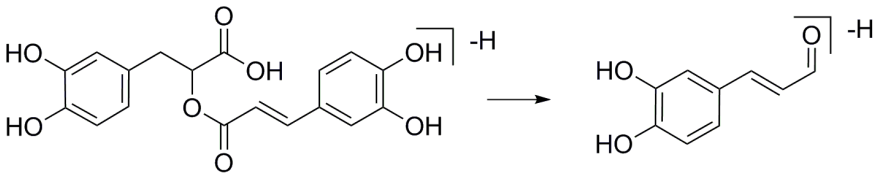
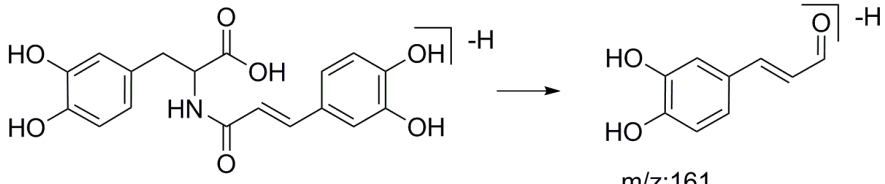




(a)



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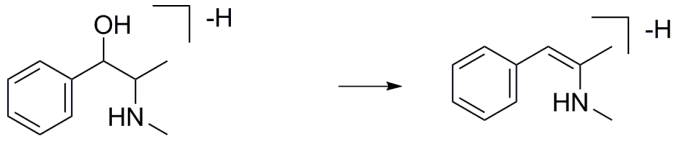

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