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Original Article

LC-UV method to assay raloxifene hydrochloride in rat plasma and its application to a pharmacokinetic study

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A specific, precise, and accurate LC-UV method was developed and validated to assay raloxifene hydrochloride in rat plasma. Raloxifene was analyzed after liquid-liquid extraction and quantified by reversed phase liquid chromatography (C18 column) using acetonitrile and ammonium acetate buffer 0.05 M (pH 4.0) as mobile phase at a flow rate of 1 mL.min^{-1} and UV detection at 287 nm. Retention times of raloxifene and internal standard (dexamethasone) were approximately 11 min and 14 min, respectively. Linearity was checked for a concentration range between 25 ng.mL^{-1} and 1000 ng.mL^{-1}. Intra- and inter-day precision had relative standard deviation lower than 10% and 15%, respectively. Recovery from plasma was higher than 90%. Accuracy values were 98.21%, 99.70%, and 102.70% for lower, medium, and upper limits of quantification, respectively. Limit of quantification was 25 ng.mL^{-1}. Drug stability was analyzed at room temperature using plasma kept in a freezer at -80 °C for 45 days after processing for 6 h and three freeze-thaw cycles. The advantages of the method developed include stability under different conditions and low limit of quantification. Its applicability was confirmed by the analysis of raloxifene levels in plasma samples in a designed pharmacokinetic study in rats after intravenous administration (5 mg.kg^{-1}).

Keywords: Raloxifene/pharmacokinetic; Liquid chromatography. Plasma. Bioavailability.

INTRODUCTION

Characterized by reduced bone mass, osteoporosis affects women in the menopausal period. Raloxifene hydrochloride (RH; $C_{28}H_{27}NO_4S.HCl$) is a selective estrogen receptor modulator with agonist effect on bones. It is usually administered to women for the prevention and treatment of osteoporosis (Morello, Wurz, DeGregorio, 2003; Kayath, 1999). A 60-mg RH tablet is administered orally, when 60% of the dose is absorbed from the gastrointestinal tract and reaches absolute bioavailability of mere 2% (Morello, Wurz, DeGregorio, 2003). With an extensive intestinal and hepatic metabolism, RH has a half-life of 28 h (Kosaka *et al*., 2011; Jeong *et al*., 2005). The compound is extensively distributed in the body. Volume of distribution is not dose-dependent, and RH is highly bound to plasma proteins [98-99%] (Morello, Wurz, DeGregorio, 2003; Hochner-Celnikier, 1999). Most of the RH dose administered and the main part of its glucoronide metabolites are excreted in feces (Hochner-Celnikier, 1999).

The quantification of RH in plasma has been described using different techniques, such as capillary electrophoresis (Pérez-Ruiz, 2004), liquid chromatography coupled to mass spectrometry (Trontelj *et al.*, 2007), and ultra performance liquid chromatography (Jadhav, Ramaa, 2012). Few articles have discussed the development and validation of an analytical method for the quantification of RH in rat plasma using liquid chromatography with ultraviolet detection (LC-UV). Nevertheless, the applicability of this technique has been investigated in the quantification of RH in drug dosage forms (Salazar *et al*., 2015). Yang *et al.* (2007) validated a LC-UV method to determine raloxifene in rat plasma in a pharmacokinetic study, reporting a limit of quantification of 0.20 µg.mL^{-1}

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using 23 ºC as column temperature. On the other hand, Chen *et al.* (2010) conducted a RH pharmacokinetic study in rats based on a LC-UV method using a gradient elution of the mobile phase and a limit of quantification of 0.56 $\mu g.mL^{-1}$. However, to the best of our knowledge, simple and inexpensive pharmacokinetic analytical methods with low limits of quantification and excellent accuracy for the analysis of RH in biological samples have not been designed. In addition, Ravi, Aditya and Vats (2012) developed a LC-UV method to estimate RH levels in rabbit plasma with a limit of quantification of 0.05 $\mu g.mL^{-1}$. It is important to highlight that these methods (Yang *et al.*, 2007; Chen *et al.*, 2010; Ravi, Aditya, Vats, 2012) were based on a protein precipitation technique to extract the drug from rat or rabbit plasma.

In this scenario, the aims of this study were to develop and validate an analytical LC-UV method based on liquid-liquid technique to extract the drug from rat plasma. This approach was proposed to afford low limits of quantification to assay RH in rat plasma, considering the requirements for suitable application in a pharmacokinetic study after RH intravenous administration.

MATERIAL AND METHODS

Material

Briefly, RH was obtained from Sequoia Research Products (Oxford, United Kingdom). Dexamethasone (internal standard - IS) was donated by Multilab Industry of Pharmaceutical Products Ltda (São Jerônimo, Brazil). Ammonium acetate was supplied by Stilolab Products for Laboratory (Porto Alegre, Brazil). Acetonitrile and methyl tert-butyl ether (MTBE) HPLC grade were purchased from Tedia (Rio de Janeiro, Brazil).

Apparatus and chromatographic conditions

An HPLC apparatus was used to carry out the validation study. The system consisted of a liquid chromatograph (10AD model, Shimadzu, Japan) with a SPD-M20AV detector, a degasser DGU-20A5, a CBM-20A controller, a LC-20AT pump, and a SIL-20A auto sampler. Chromatographic separation was performed in a C18 column (150 mm x 4.6 mm, 5-µm particle size, 110-Å pore diameter; Discovery®, Supelco Analytical, Sigma-Aldrich) and a mobile phase composed of acetonitrile 95% and 0.05 M ammonium acetate (28:72 v/v) containing 0.2% glacial acetic acid at isocratic flow rate (1.0 $mL.min^{-1}$). The mobile phase was filtered using a membrane (0.45 µm, Millipore®) and a vacuum pump, and degassed before use. The analysis was performed for 18 min at 287 nm with injection volume of 40 µL. The ratio of peak area of RH to IS was used for the quantification of plasma samples.

Preparation of standard solutions

A RH stock solution was prepared dissolving 12.5 mg of the drug in methanol in a volumetric flask (25 mL) to a final concentration of 0.5 $mg.mL^{-1}$. This solution was stored in a freezer at -80 °C and was diluted with mobile phase to obtain a 50.0 $\mu g.mL^{-1}$ solution, immediately before use. Working standard solutions were prepared daily by serial dilution of the solution at 50.0 $\mu g.mL^{-1}$ with the mobile phase to obtain analyte concentrations of 0.25, 0.50, 1.00, 2.50, 5.00, and 10.00 $\mu g.mL^{-1}$. The internal standard (dexamethasone) working solution was prepared in methanol to yield a concentration of 0.50 $mg.mL^{-1}$ and diluted to 100.0 $\mu g.mL^{-1}$ immediately before use.

The quality control plasma samples (QC) were prepared from a different stock solution from that used to generate the analytical curve samples using working standard solutions at 0.35, 4.50, and 9.00 $\mu g.mL^{-1}$. These samples were used to evaluate intra- and inter-day variations.

Standard solutions in plasma

The samples of analytical curves (25.0, 50.0, 100.0, 250.0, 500.0, and 1,000 $ng.mL^{-1}$) were prepared using 90 µL of blank rat plasma with 10 µL of internal standard and 10 µL of working standard solutions. The final concentration of internal standard was 10 $\mu g.mL^{-1}$.

Sample preparation

Plasma samples were stored in a freezer at -80 °C upon analysis (for approximately 45 days). The liquid-liquid technique was used to extract the drug from plasma. In an Eppendorf tube, 10 µL of internal standard was added to 100 µL of plasma samples, followed by vortex mixing for 5 s. The organic solvent MTBE (500 µL) was added for the extraction. Samples were shaken (10 min) and centrifuged (6800 *g* for 10 min at 4 °C). The supernatants were transferred to tubes for evaporation of the organic solvent in a centrifuge at 40 °C. The pellets were resuspended with mobile phase (100 µL), stirred for 5 s (vortex mixing), sonicated for 10 min, and assayed following the LC-UV method described above.

Bioanalytical method validation

The method was validated according to the FDA Guidance – Bioanalytical Method Validation (2001) considering linearity, lower limit of quantification, specificity, precision, accuracy, and stability.

Linearity, lower limit of quantitation and specificity

To analyze linearity, six analytical curves prepared using six plasma concentrations in the range of 25.0 ng.mL^{-1} to 1,000 ng.mL^{-1} (25.0, 50.0, 100.0, 250.0, 500.0, and 1,000 ng.mL^{-1}) were assayed on two consecutive days. The drug was extracted from the biological samples and resuspended in 100 µL of the mobile phase according to the technique described in the "Sample preparation" section, followed by the LC-UV analysis. Each analytical curve was plotted using the average relative area of each concentration of the curve (peak area of RH/peak area of IS). The six concentrations of the standard solution were analyzed by linear regression to calculate the equation of the calibration curve and correlation coefficients. The lowest concentration of the analytical curve with acceptable precision and accuracy was considered the limit of quantification. Specificity of the method was analyzed assaying six blank plasma samples from rats.

Precision and accuracy

Intra- and inter-day precision as well as accuracy of the analytical method were determined calculating the standard deviation (SD) and relative standard deviation (RSD%) of three concentrations (low, medium, and high) in six replicate analysis. Samples were prepared with 80 µL, 10 µL of the internal standard solution (10 µg.mL^{-1}), and 10 µL of RH standard solution. The drug was extracted and resuspended in 100 µL of the mobile phase to reach concentrations of 35.0, 450.0, and 900 ng.mL-1 of RH as described above.

Recovery

Relative recovery was analyzed comparing the analytical results of extracted samples from three concentrations (35.0, 450.0, and 900 ng.mL^{-1}) with those obtained from standard solution that is 100% of recovery.

Stability

The stability of RH in rat plasma was performed using the low (35 ng.mL^{-1}) and high (900 ng.mL^{-1}) concentrations. The samples were analyzed in the short-term (6 h on the workbench) after processing (6 h in autosampler), after three freeze-thaw cycles, and after long-term storage in freezer at -80 °C (45 days).

Pharmacokinetic study

A pilot pharmacokinetic study was performed using male Wistar rats (n = 3), and the analytical method validated was evaluated to determine RH plasma concentration. The experiment was performed according to the Severity Guide of Scientific Procedures and as approved by Ethics Committee on Animal Use of the Federal University of Rio Grande do Sul (Protocol number 22226).

Three rats (250-350 g) were purchased from the Center for Reproduction and Experimentation of Laboratory Animal of Federal University of Rio Grande do Sul. The animals were exposed to daily 12-h dark-light cycles in a room with controlled temperature (22 ± 1 °C), relative humidity of approximately 65%, and were offered water and food *ad libitum*.

On the day of the experiment, rats were anesthetized with urethane (1.25 g.kg^{-1}) and the carotid artery was exposed for cannulation and subsequent blood collection (200 µL). A solution of RH (2.5 mg.mL^{-1}) was prepared dissolving 12.5 mg of drug in 5 mL of glucose solution containing 5% of dimethylsulfoxide. This solution was administered as a 5-mg.kg^{-1} intravenous dose in the femoral vein.

Blood samples were collected at time zero (before administration of drug) and 0.08, 0.17, 0.25, 0.5, 1.0, 3.0, 6.0, 9.0, and 12.0 h after intravenous administration. Samples were centrifuged at 4 °C to separate the plasma (6800 *g* for 10 min) and stored in a freezer (-80 °C) upon analysis using the validated method described above. Plasma pharmacokinetic profiles of RH were analyzed by non-compartment approach using the Phoenix® Software (Certara, 2015, USA).

RESULTS AND DISCUSSION

Chromatograms of blank rat plasma and rat plasma containing RH (500 ng.mL^{-1}) with internal standard (10 µg.mL^{-1}) are shown in Figure 1. The chromatograms confirm specificity of the method, since it was possible to separate RH and internal standard peaks from endogenous substances of plasma. The mean retention times of RH and internal standard were approximately 11 min and 14 min, respectively. Endogenous substances were detected until 9 min into the run time of 18 min.

The mean analytical curve (25-1000 ng.mL^{-1}) showed adequate linearity (r = 0.9986 ± 0.0012), angular coefficient (slope) of 0.0017 ± 0.0001, and an intercept of 0.0190 ± 0.0167. The limit of quantification was set at 25 ng.mL^{-1} of RH (which showed intra- and inter-

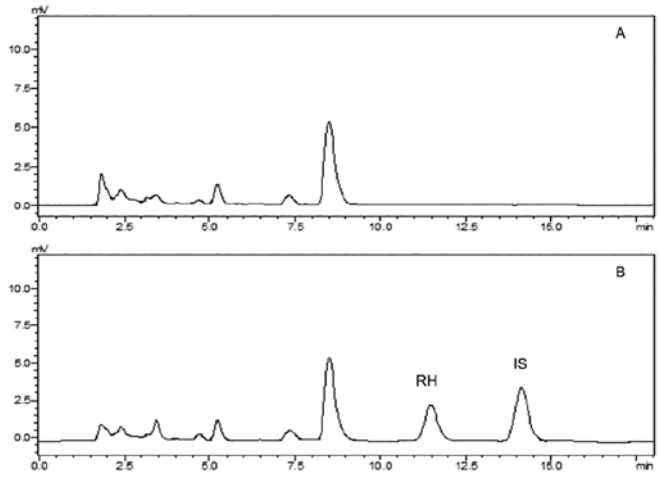

FIGURE 1 - Chromatogram obtained for (A) blank plasma, (B) blank plasma with raloxifene hydrochloride at 500 ng.mL^{-1} (RH), and internal standard 10 µg.mL^{-1} – (IS).

day imprecision of 10.01% and 14.08%, respectively), accuracy of 96.19 ± 7.03%, and a recovery of 90.12 ± 5.34%. These values are in agreement with the FDA guidelines (FDA Guidance – Bioanalytical Method Validation, 2001). Moreover, this limit of quantification value was lower than other limits of quantification previously reported in the literature for the RH assay in biological samples by LC-UV methods (Yang *et al.*, 2007; Chen *et al.*, 2010; Ravi, Aditya, Vats, 2012), which can be explained considering the liquid-liquid extraction technique used in this study, compared with the methods of simple protein precipitation from rat (Yang *et al.*, 2007; Chen *et al.*, 2010) or rabbit (Ravi, Aditya, Vats, 2012) plasma used in previous studies.

Table I shows the results of precision (RSD), accuracy (%), and recovery (%) obtained in the analysis of samples containing RH 35 ng.mL^{-1}, 450 ng.mL^{-1}, and 900 ng.mL^{-1} (low, medium, and high concentration, respectively).

The RSD results, comprising repeatability (intra-day precision), and intermediate precision (inter-day precision) were lower than 10% and 15%, respectively. Accuracy was approximately 100%. Recovery, which represents extraction efficiency, was higher than 90% for the three concentrations, in accordance with previous reports (Yang *et al.*, 2007). These results also agree with International Guidelines (FDA Guidance – Bioanalytical Method Validation, 2001).

The stability of the samples was studied according to the working conditions, using RH concentrations of 35 ng.mL^{-1}and 900 ng.mL^{-1}. The results are shown in Table II. The maximum RSD of 8% demonstrates the stability of the analyte throughout sample processing. These results confirmed all requirements for a bioanalytical method according to international guidelines. Therefore, the method developed was tested again in a RH pharmacokinetic study in order to highlight its importance and contribution to pharmacokinetics.

TABLE I - Intra- and inter-day precision (RSD), accuracy (mean ± SD) and relative recovery (mean ± SD) for the RH quality controls in rat plasma (n = 6)

Concentration (ng.mL^{-1})	Intra-day precision (RSD, %)	Inter-day precision (RSD, %)	Accuracy (%)	Recovery (%)
35	9.68	14.84	98.21 ± 8.49	91.45 ± 4.62
450	2.44	7.97	99.70 ± 7.89	94.21 ± 3.38
900	8.87	8.62	102.70 ± 8.76	92.10 ± 6.53

TABLE II - Stability of RH in rat plasma at two quality control levels (n = 3)

Condition	Concentration (ng.mL^{-1})	RH content ± SD (%)	RSD (%)
Short-term	35	100.52 ± 4.66	4.64
	900	104.83 ± 8.39	8.00
Autosampler	35	99.26 ± 4.00	4.03
	900	100.08 ± 5.98	6.12
Freeze-thaw	35	101.74 ± 5.90	6.09
	900	97.05 ± 7.18	7.46
Long-term	35	102.76 ± 4.78	4.99
	900	97.89 ± 5.84	5.99

Figure 2 shows the plasma pharmacokinetic profile after intravenous administration of RH 5 mg.kg^{-1} (n = 3). A rapid distribution of the drug from plasma was observed. The pharmacokinetic parameters obtained after non-compartmental analysis were: $AUC_{0-\infty}$ = 1924.32 ± 264.42 ng.h.mL^{-1}, λ = 0.20 ± 0.01 h^{-1}, $t_{1/2}$ = 3.45 ± 0.23 h, MRT = 4.52 ± 0.17 h, CL = 2.94 ± 0.43 L.kg.h^{-1}, Vdss = 13.26 ± 1.36 L.Kg^{-1}. Furthermore, the analytical method showed sufficient sensitivity to detect very low RH plasma concentration, even at the last phase of the pharmacokinetic profile, when as little as 25 ng.mL^{-1} of drug was quantified. It would not be able to be detect such a low concentration of RH using LC-UV methods previously described in the literature to estimate the amounts of the drug in plasma.

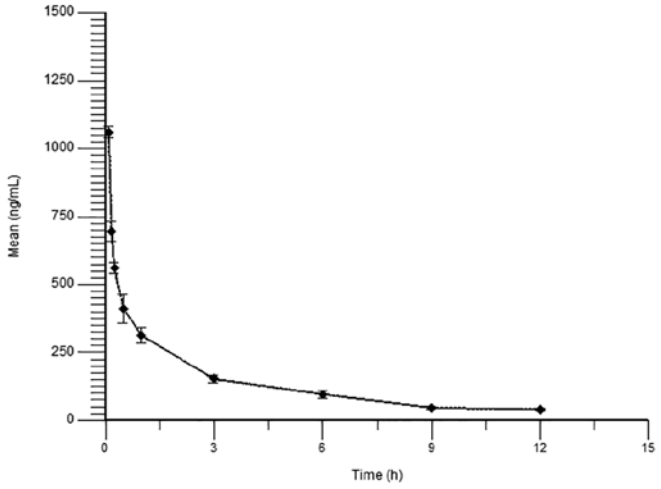

FIGURE 2 - Mean plasma concentration-time profile of RH after intravenous administration of 5 mg.kg^{-1} drug. Data are expressed as mean ± SD (n=3).

The lack of methods with low limits of quantification of RH in rat plasma may be the reason why previous pharmacokinetic studies evaluated only relative bioavailability (Yang *et al.*, 2007; Chen *et al.*, 2010) due to the methodological restrictions to assay RH in the last phase.

CONCLUSION

A method to assay RH in rat plasma for application to pharmacokinetic studies was developed and validated using LC-UV and a liquid-liquid technique to extract the drug from plasma. This method showed good accuracy, linearity, specificity, and precision, and was successfully used to analyze rat plasma samples in a pilot pharmacokinetic study. The validated method has notable advantages, like the lower limit of quantitation compared with other LC-UV methods previously described in the literature, a good resolution between the analyte and the plasma components, and RH stability under different conditions. The present analytical method is suitable for use in pharmacokinetic studies that require the quantification of very low concentrations of drug in plasma, such as studies involving the development of innovative formulations containing RH.

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