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Development, Validation and Pharmacokinetic Application of a Simple and Robust RP-HPLC Method for Quantitation of Raloxifene in Rat Plasma

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***SUMMARY*. A simple and sensitive HPLC method has been developed and validated for the quantification of raloxifene in rat plasma. Liquid- liquid extraction procedure was employed for extracting raloxifene from rat plasma sample. Separation of raloxifene was achieved through a RP- C18 column with a mobile phase consisted of phosphate buffer and acetonitrile (66:34 %, v/v). The method was validated for specificity, selectivity, sensitivity, linearity, accuracy, precision, recovery and stability parameters. A linear response (R2 value 0.9991) was found over the calibration range of 50 to 500 ng/mL. The accuracy for intra and inter day run varied between 86.73 to 102.30 % and 91.11 to 95.56%, respectively. The corresponding precision (% CV) were within 0.82 to 9.43% and 6.23 to 8.33%. The method was also found to be specific and stable. The applicability of the method was established through a single dose oral pharmacokinetic study of raloxifene in rat.**

RESUMEN. Se ha desarrollado y validado un método de HPLC simple y sensible para la cuantificación de raloxifeno en plasma de rata. Se empleó un procedimiento de extracción líquido-líquido para extraer raloxifeno de la muestra de plasma de rata. La separación xifeno se consiguió a través de una columna RP-C18 con una fase móvil constituida por tampón fosfato y acetonitrilo (66:34%, v/v). El método fue validado para los parámetros de especificidad, selectividad, sensibilidad, linealidad, precisión, seguidad, recuperación y estabilidad. Se encontró una respuesta lineal (valor R2 = 0,9991) en el intervalo de calibración de 50 a 500 ng/mL. La exactitud para intra- e inter día varió entre 86.73 a 102.30% y 91.11 a 95.56%, respectivamente. La precisión correspondiente (% CV) fue de 0,82 a 9,43% y de 6,23 a 8,33%. También se encontró que el método era específico y estable. Se estableció la aplicabilidad del método mediante un estudio farmacocinético de dosis única oral de raloxifeno en ratas.

INTRODUCTION

Raloxifene is a non-steroidal selective estrogen receptor modulator and chemically belongs to the benzothiophene class. It is used for the hormone replacement therapy in post-menopausal women to normalize the condition of climacteric symptoms. Raloxifene helps in maintaining the bone density and normal bone histology in post-menopausal women. It also reduces the chances of coronary heart diseases [1-3]. Raloxifene HCl shows agonist effects on the liver and bone while anti-estrogenic effect on breast and uterus tissue [4]. It is supplied as 60 mg conventional tablets administered daily as a single dose. It shows a low solubility and high permeability, and belongs to the Biopharmaceutics Classification System (BCS) Class II. It is rapidly bio-transformed with a serum half -life of 27.7 hours [5]. Oral bioavailability of raloxifene is very low and limited up to 2% [6].

Different types of research, including development of newer dosage forms for raloxifene shows a growing interest nowadays for the researcher. All those developed formulations need to be evaluated for their pharmacokinetic profile in animals. Although, several bioanalytical meth-

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ods have been reported for quantitation of raloxifene in animal plasma [7-11], the methods reported till date suffers from several limitations such as lack of sensitivity, long run time, complicated sample preparation procedure, requirement of the high volume of plasma samples etc. Some methods require isotopic detection [12,13]. The methods are either not reproducible or shows a high degree of interference. Few liquid chromatography mass spectrometry (LCMS) method has been developed for quantitation of raloxifene as well. The LCMS method developed by Chen *et al.* [14] and Trdan *et al.* [15] is specifically useful to quantitate raloxifene in human urine. But the method cannot be utilized to determine the concentration of raloxifene in rat plasma because of the difference in biological species as well as type of matrix. Again, an LCMS method has been reported by Trontelj *et al.* [16] for raloxifene quantification in human plasma. The method followed solid phase extraction (SPE) procedure for extraction of raloxifene from human plasma. The SPE is a complicated phenomenon and requires specialized instrumental set up. The SPE cartridges also can not be re-use. As a whole, the procedure is not economical to the researcher. Moreover, LCMS is a costly instrument and the majority of the general laboratories can not afford to buy LCMS and are normally equipped with the comparatively less costly instrument HPLC. Most importantly, as per the United State Food and Drug Administration (USFDA) guideline, when there is a change in the matrix within species (*e.g.,* human plasma to human urine) or change in species within the matrix (*e.g.,* rat plasma to human plasma), the method should be considered as new and validation have to be performed before its intended use [17]. Therefore, the above mentioned LCMS methods are not applicable for determination of raloxifene content in rat plasma.

Consideration of all these factors directs us to develop a new superior alternate bioanalytical method for raloxifene quantification in rat plasma. The aim of this research was to develop and validate a simple, specific, sensitive, accurate and reproducible method for quantitation of raloxifene in rat plasma and evaluation of its applicability in pharmacokinetic study.

MATERIAL AND METHODS

Chemicals and reagents

Raloxifene HCl (Fig. 1) was purchased from Binzhou Neophar Pharmaceutical Co. LTD, China. The certified purity of raloxifene was 99.4%. The internal standard (IS) diclofenac sodium (Fig. 1) was purchased from CCM Pharmaceutical SDN BHD, Malaysia. Acetonitrile, HPLC grade water and potassium dihydrogen phosphate was purchased from Fisher scientific, Malaysia. All other chemicals used were of analytical reagent grade. Blank rat plasma was collected from the healthy rats through cardiac puncture. Briefly, the animals were anesthetized with sodium pentobarbital, and cardiac puncture was rapidly performed. The rat blood was then collected into the tubes containing EDTA as anticoagulant. The plasma was separated by centrifugation at 5,000 rpm (4 °C) for 10 min and stored at– 40 °C until use.

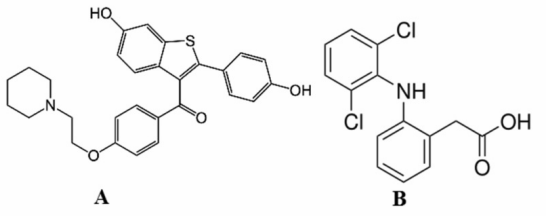

Figure 1. Structural representation of (**A**) raloxifene and (**B**) diclofenac.

Instrumentation and chromatographic conditions

The high performance liquid chromatography (HPLC) system consisted SHIMADZU LC-20ÂT low pressure gradient solvent delivery segment (SHIMADZU, Kyoto, Japan) equipped with DGU-20-A512 degassing unit, SPDM20A Prominence Diode array UV/VIS detector, SIL-20AHT auto sampler, CTO-10AS VP column oven and LC workstation data processor. As a part of the method development process, a wide verity of mobile phase composition and columns were tried for determining the suitability of the analysis. The method was optimized through changing the ratio of the organic and aqueous solvent, mobile phase pH, column and other chromatographic parameters. The final selection was based on the optimum resolution, peak shape and total chromatographic run time of the method. The chromatographic separation of raloxifene was achieved through a thermofisher syncronis RP- C-18 column (15 × 4.6 mm, 5 µm pore size) with a mobile phase consisted of a mixture of phosphate buffer and acetonitrile (66:34) at pH 6.1. The column oven temperature was required to maintain at 30 °C. The

flow rate was set at 1.0 mL/min and the analyte was detected at a wavelength of 285 nm. The sample injection loop volume was adjusted to 20 µL. LC lab solution software was used for the HPLC system.

Preparation of calibration standards and quality control samples

The calibration standards for linearity study were prepared after spiking the drug solution in the blank rat plasma. Prior to the extraction, plasma samples were treated with 100 µL of 0.1 N HCl solutions for acidification. An IS stock solution of diclofenac sodium at a concentration of 2000 ng/mL was prepared by dissolving it in a solvent mixture of water and methanol (4:1). A volume of 2 µL of this IS stock solution was added to the spiked rat plasma. Six sets of calibration standards at different concentrations of 50, 100, 150, 200, 300, and 500 ng/mL were prepared. The method was validated at three different quality control levels. The low quality control (LQC), mid quality control (MQC) and high quality control (HQC) solutions were prepared by spiking raloxifene HCl solution in blank rat plasma to produce the final concentrations of 75, 250, and 400 ng/mL, respectively.

Sample extraction procedure

The analyte was extracted from the plasma samples following liquid-liquid extraction procedure using ethyl-acetate as an extracting solvent. An amount of 0.45 mL of blank rat plasma was taken in a glass tube and appropriate amount of raloxifene HCl solution was spiked into it. To this, 2 µL of the internal standard solution was added and mixed well. A volume of 4 mL ethyl-acetate was added to the plasma solution. This mixture was then vortex mixed for about 45 s and centrifuged for 20 min at 4000 rpm. The supernatant layer (3.2 mL) was collected and evaporated to complete dryness using gentle stream of nitrogen at a temperature not exceeding 50 °C. The dried extract was then reconstituted with 300 µL of acetonitrile, filtered with 0.22 µm membrane filter and injected into the HPLC system.

Method validation

The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation of USFDA [17-19].

Specificity and selectivity

Specificity and selectivity of the method were determined to confirm the ability of the developed method to differentiate and quantify the analyte in the presence of other components in the sample. The extracted blank plasma sample was run and analysed to determine at which extent the internal plasma components contribute to the retention time of raloxifene and diclofenac sodium (IS). Six different batches of rat plasma were analysed for selectivity and specificity with the specified chromatographic conditions.

Linearity

Six calibration standards with different concentrations were prepared in the range of 50 to 500 ng/mL together with IS. The samples were extracted by liquid-liquid extraction method as described. A standard curve was constructed by plotting the peak area ratio between raloxifene and diclofenac sodium (IS) at Y-axis against the corresponding concentration of raloxifene at X-axis. Linearity was expressed by the experimentally determined coefficient of correlation value.

Limit of quantification and limit of detection

Limit of quantification (LOQ) was calculated from the minimum concentration of the calibration curve, which was detected and quantified with a CV% ≤ 20. The limit of detection (LOD) was the minimum concentration of the analyte present in detectable amount. LOD and LOQ were determined from an area ratio of peak and noise level (S/N) as three and ten, respectively.

Accuracy and precision

Precession and accuracy of the method was determined after comparison of the analytical data from intra-day and inter-day run on three levels of quality control samples (LQC, MQC and HQC). Six replicates of all the three concentrations were quantified to determine the intra-day and inter-day precision and accuracy of the developed method. Peak areas were calculated for relative standard deviation (RSD) for determining the precision (%CV) of the method.

Extraction Recovery

Extraction recovery of raloxifene HCl from the rat plasma was determined from six replicates of LQC, MQC and HQC samples. The measured peak area was compared with the same concentration of raw samples containing the same concentration of the raloxifene HCl as 100%.

Robustness

Robustness was evaluated on the developed HPLC method by running the QC samples at

flow rate 0.9 and 1.1 mL/min. The mean recoveries of the samples were calculated and compared against that of the samples run at flow rate 1.0 mL/min.

Stability studies

Stability of raloxifene in plasma samples was determined after performing a short term stability study for 12 h, freeze-thaw stability study for three freeze thaw cycles and long term stability study for 30 days. Blank rat plasma was spiked with raloxifene at the concentration of three quality control level in triplicate. The plasma samples were extracted and analysed after storage required for the individual stability tests.

For short term stability test, blank plasma samples were spiked with raloxifene at three quality control level and kept at room (22-25 °C) temperature for 12 h, extracted as described above and analysed. For freeze thaw stability study, QC samples were subjected to 3 consecutive freeze (-20 °C) thaw cycles. In long term stability the processed QC samples were kept at -20 °C for 30 days. The standard calibration curve was developed separately from the each stability study performed and accuracy of the stability samples was determined.

Pharmacokinetic application study

The validated bioanalytical method was used in pharmacokinetic evaluation on Sprague-Dawley rats. The study was approved by the animal ethical committee of International Islamic university Malaysia (Reference no: IIUM/519/14/4/IACUC). A total number of 6 rats were used in the study. Animals were kept under good laboratory conditions (23 ± 2 °C; 45 ± 6% relative humidity) and were exposed to dark and light cycle (12 h/12 h) for 7 days before the experiment to adjust them with the environmental condition. Throughout this period, they provided sufficient standard dry pellet diet and water. Raloxifene tablets Evista (Elly Lilly) were purchased and used in the study. A calculated amount of drug was taken and studied for its bioavailability. On the day of dosing, raloxifene was administered orally to the rats (6 mg, individually). Blood samples were collected at a time interval of 0, 2, 4, 8, 12, 24, and 48 h into the collection tubes containing EDTA-K3 as an anti-coagulant. Plasma was separated by centrifuging the blood and stored frozen at -20°C until analysis. To the 0.45 mL of plasma sample, IS solution was added, vortex mixed, extracted and analysed for determination of raloxifene content using the developed HPLC method. To evaluate the pharmacokinetic parameters, area under the plasma-concentration–time curve from time zero to the last measurable raloxifene sample time and to infinity ($AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$), maximum plasma concentration (C_{max}), time to reach maximum plasma concentration (T_{max}), elimination rate constant (Kel) and elimination half-life ($t_{1/2}$) were determined for the period of 0 to 48 h by non-compartmental method after the oral dosage.

RESULTS AND DISCUSSION

A simple and sensitive HPLC method for determination of raloxifene in rat plasma was developed and validated as per the USFDA guideline.

Selectivity and specificity

The developed HPLC method was found to be selective and specific as it was able to differentiate and quantify raloxifene in the presence of other plasma component and the IS. A series of molecules were screened for their suitability to be used as IS in this method. Amongst them, diclofenac was selected because of its adequate resolution with raloxifene, satisfactory peak shapes, stability and consistency in area count during the analysis. The blank plasma chromatogram is shown in Fig. 2. No interference was observed at the retention time of raloxifene and diclofenac sodium (IS). The chromatogram of the same showed good separation with low background noise. The retention time for diclofenac sodium and raloxifene were about 6.9 min and 10.8 min, respectively. The total chromatographic run time was 12.0 min (Fig. 3).

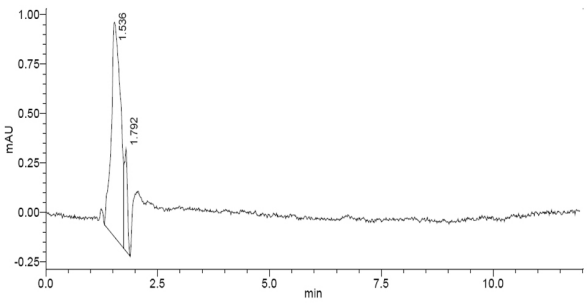

Figure 2. Typical HPLC chromatograms of blank rat plasma.

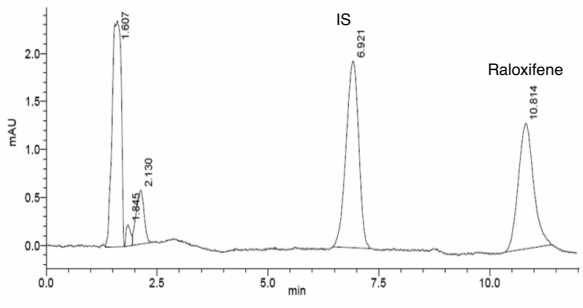

Figure 3. Typical HPLC chromatograms of raloxifene spiked in rat plasma with IS.

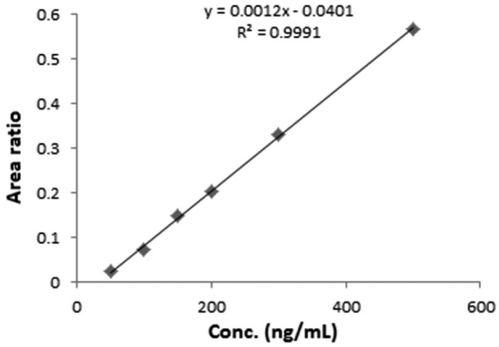

Figure 4. Calibration curve for raloxifene.

Linearity

The method was found to be linear over the calibration range of 50 to 500 ng/mL concentration. Fig. 4 shows the calibration curve which has a correlation coefficient (R^2) value of 0.9991. This indicated an excellent correlation between peak area ratios and concentration of raloxifene.

Limit of quantification and limit of detection

The minimum detectable concentration (LOD) of raloxifene was found to be 16 ng/mL, whereas the limit of quantification (LOQ) was 48 ng/mL.

Accuracy and precision

As per the USFDA guidelines for bioanalytical method validation, the mean value should be within 15% of the nominal value. The deviation of the mean from the nominal value serves as the measure of accuracy. Again, the precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV). The intra-day and inter-day accuracy-precision results are summarized in Tables 1 and 2, respectively. The accuracy of this bioanalytical method for intra and inter day run varied between 86.73 to 102.30% and 91.11 to 95.56%, respectively. The corresponding precision (%CV) for intra and inter day run were within 0.82 to 9.43% and 6.23 to 8.33%. Therefore, the accuracy and precision of the method evaluated at three quality control level met the acceptance criteria.

Extraction recovery

The recovery of raloxifene from the plasma was evaluated at three quality control level were found to be 93.44% (LQC), 96.42% (MQC) and 92.75% (HQC). Hence, the developed liquid-liquid extraction procedure is good enough to extract the analyte from the rat plasma samples.

Quality control	Run	Measured concentration of raloxifene (ng/mL)			
		Mean	SD	% CV	% Accuracy
LQC	1	69.87	1.30	1.86	93.16
	2	70.12	5.10	7.28	93.50
	3	65.05	3.53	5.44	86.73
MQC	1	227.31	9.86	4.33	90.92
	2	255.75	24.13	9.43	102.30
	3	242.85	20.87	8.53	97.14
HQC	1	379.22	20.67	5.45	94.80
	2	382.82	3.13	0.82	95.70
	3	352.94	23.70	6.71	88.23

Table 1. Intra-day precision data of the analytes. % CV (precision); coefficient of variation, Accuracy: (Mean assayed concentration – nominal concentration)/ (nominal concentration) × 100.

Quality control	Measured concentration of raloxifene (ng/mL)			
	Mean	SD	% CV	% Accuracy
LQC	68.33	4.30	6.23	91.11
MQC	238.92	19.91	8.33	95.56
HQC	371.58	22.66	6.09	92.89

Table 2. Inter-day precision data of the analytes. % CV: Coefficient of variation (SD x 100/Mean), Accuracy: (Mean assayed concentration – nominal concentration)/ (nominal concentration) × 100.

Quality control	Stability	Measured concentration of raloxifene (ng/mL)			
		Mean	SD	% CV	% Accuracy
LQC	0 h - autosampler	70.08	3.29	4.70	93.44
	12 h -autosampler	70.13	3.63	5.17	93.74
	12 h -bench top	72.06	1.86	2.59	96.09
	3rd freeze/thaw	70.17	5.10	7.28	93.50
	30 day at -20 °C	65.85	1.33	2.03	87.80
MQC	0 h - autosampler	241.07	22.93	9.51	96.42
	12 h -autosampler	249.30	21.38	8.57	99.72
	12 h -bench top	236.42	18.54	7.84	94.57
	3rd freeze/thaw	247.45	2.28	0.92	98.98
	30 day at -20°C	224.04	8.82	3.93	89.93
HQC	0 h - autosampler	371.02	24.27	6.54	92.75
	12 h -autosampler	367.88	22.28	6.05	91.97
HQC	12 h -bench top	386.29	8.32	2.15	96.57
	3rd freeze/thaw	383.36	8.68	2.26	95.84
	30 day at -20 °C	347.26	3.64	1.05	86.81

Table 3. Stability data of the analytes in rat plasma. % CV (precision); coefficient of variation, Accuracy: (Mean assayed concentration – nominal concentration)/ (nominal concentration) × 100.

Robustness

The robustness of the developed HPLC method was determined by running QC samples at two different flow rates of the mobile phase. At 0.9 mL/min flow the accuracy was found to be within 87.61 and 97.27%. Again, at a flow rate of 1.1 mL/min, the accuracy was found to be within 93.04 and 99.48%.

Stability

The stability data for validation of the method has been summarized in Table 3. The mean accuracy of the analyte after keeping the QC samples for short term on bench top for 12 h at room temperature were 96.90, 94.57 and 96.57%, respectively. The analyte was found stable inside the autosampler for 12 h, as the accuracy of the QC samples were 93.74% (LQC), 99.72 (MQC) and 91.97% (HQC). After three freeze thaw cycle the accuracy of the QC samples were 93.50, 98.98 and 95.84% for LQC, MQC and HQC respectively. Raloxifene was found to be stable in rat plasma for at least 30 days at -20°C as the measured accuracy were 87.80, 89.93 and 84.60 for LQC, MQC and HQC respectively. Therefore, the method showed excellent stability in the all tested stability conditions.

Pharmacokinetic application study

The developed bioanalytical method was used to analyse the plasma samples of a pharmacokinetic study of raloxifene in rat. The maximum plasma concentration (C_{max}) of raloxifene was 140.14 ± 23.18 at 5.33 ± 2.30 h (T_{max}). The plasma half-life was found to be 22.29 ± 9.5 h. The mean value of area under curve the concentration time (AUC_{0-t}) obtained was 2841.18 ± 1019.92 ng h/ml and area with infinite time (AUC_{0-inf}) was 4045.95 ± 2290.42 ng h/ml. Profiles of the mean plasma concentration of raloxifene over time are shown in Fig. 5.

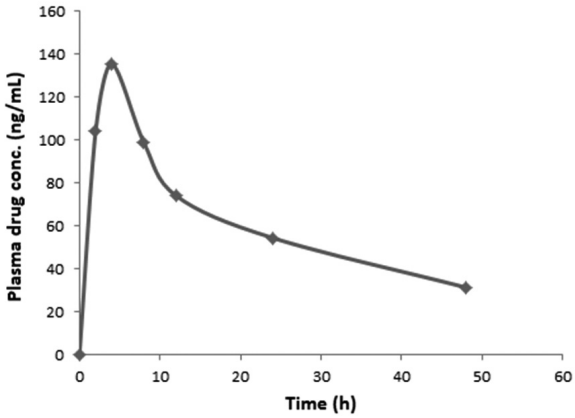

Figure 5. Plasma concentration–time profile of raloxifene in rat plasma following oral administration.

CONCLUSION

In this study, a simple, sensitive, accurate, precise and stable HPLC method has been developed and validated for quantitation of raloxifene in rat plasma. The use of liquid-liquid extraction method resulted in the increased recovery of the raloxifene in the plasma when compared with protein precipitation method reported previously. Samples extracted with protein precipitation method may contain a high amount of plasma components which have all the possibility to interfere during the entire chromatographies procedure and detection technique. Again, due to high load of plasma component, there is a high chance of contamination of the HPLC system including injector, column and detector. The self-life of column thus may decrease. Injection of plasma samples extracted by liquid liquid extraction does not suffer from such limitations. This method is thus can be considered as more advantageous than the previously reported methods. The use of HPLC is easy and readily available when compared with other instruments like LC-MS (Liquid-chromatography coupled with mass spectrophotometry). The method can be used to analyse the plasma samples of pharmacokinetic study or other similar type of studies involving rat plasma.

Ethical declaration. A prior approval was taken for the study from the animal ethical committee of International Islamic university Malaysia (Reference no: IIUM/519/14/4/IACUC). Animals were handled as per the institutional ethical guidelines during the entire study period.

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