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Article

Article

UPLC–MS/MS Method Validation for Estimation of Resveratrol in Rat Skin from Liposphere Gel Formulation and Its Application to Dermatokinetic Studies in Rats

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Abstract

For the quantification of resveratrol (RV) in the Wistar rat skin, an ultra-performance liquid chromatography-mass spectrometric (UPLC–MS/MS) method was developed and validated on ACQUITY UPLC BEH C_{18} column (1.7 µm). The mobile phase ratio of methanol (A) and 2% formic acid (B) (ratio 10: 90% v/v, 80: 20 v/v) at isocratic elution with flow rate 0.3 mL/min, and run time 3 min was used for analysis. In addition, the use of multiple reaction monitoring (MRM)/ES+ mode to detect the analytes and to track parents to daughter ion transition of 229.17 > 107.04 m/z (time scan 3 min, retention time 1.48) for RV and curcumin as an internal standard shows 369.16 > 176.93m/z (scan time is 2.80 min, retention time is 1.11), respectively. Linearity was observed in the range of 2.5 to 2,000 ng/mL ($R^2 = 0.987$). Precision and accuracy on rat skin were within the acceptability range (RE%: ±15; RSD%: ±15). Moreover, it showed a good percentage recovery and found within acceptance limit 90–110%. Lower limit of detection and quantitation for the method observed to be 2.5 and 20 ng.mL^{-1}, respectively. Method application indicated successful determination of dermatokinetics parameters of RV from lipospheres gel and suspension in the rats.

Introduction

Over thousands of years, herbs have been used to cure different diseases and even to enhance body function. Resveratrol (RV) in herbs identified as essential nutrients that have played an important role in enhancing health and other diseases conditions (1). It is present in fruits and in red wine as secondary metabolites. Its existence was also detected in Peanuts, Beers and Legumes in various other plant species (1). RV has two phenolic rings connected by a styrene double bond, allowing a *cis*- and *trans*-orientation of 3, 40, 5-tryhydroxystilbene to be produced (1). The grapes of the popular red grape wine are normally harvested (*Vitis vinifera*). In several medical preparations, RV has had a long tradition of serving humans around the globe. The literature has documented beneficial outcomes in the treatment or prevention of various diseases (2), including such as psoriasis, asthma, tumors, hepatoprotective, cardiovascular disorders, etc., In addition, owing to its poor solubility, higher metabolism and poor bioavailability, RV has limited therapeutic use. However, it possesses limitation for delivery through oral route of administration (1, 2). Lately, researchers developed RV-loaded gel, which decreases symptoms related to inflammatory disorders, but it produced a suboptimal therapeutic action by virtue of limited drug absorption through the skin.

In this regard, there is a great interest to increase the RV concentration into the epidermis and dermis by the liposphere-based hydrogel for skin/joint diseases, i.e. arthritis and psoriasis (3, 4). There are a range of literature available, which is validated for RV determination utilizing high-performance liquid chromatography (HPLC), UV (5), spectrofluorometric, Chemiluminescence, mass spectrometry, Gas-Chromatography-MS and adsorption-stripping voltammetry. The HPLC processes are lengthy, take around 25 min per run and involve sometimes strong step extraction to purify the extracts and eliminate interferences (5). There are many benefits to applications of liquid chromatography-tandems (ultra-performance liquid chromatography-mass spectrometric [UPLC–MS/MS]). It is an easy and non-intensive approach for application to dermatokinetics and is simple with improved precision, accuracy, sensitivity, speciality and reduced analysis time and costs (6). The equipment was often run under higher pressure (up to 15,000 psi). This method is incredibly efficient and significantly increases the overall resolution, sensitivity and analytical speed as well (6). Consequently, the UPLC–MS/MS was emerging as a strong hyphenated bioanalytical research technique (6). However, there are no sensitive bio-analytical approaches for the quantification of RV in the skin as for epidermis and dermis, to the maximum extent of our understanding. In the present text, UPLC–MS/MS has first been developed and successfully used in topical investigations to validate the quantification of RV loaded liposphere gel. In terms of its high sensitivity and very short retention time, the present approach has demonstrated excellent success in quantifying RV in the skin layer.

Materials and Method

Chemicals and reagents

RV (purity >99%; Mol wt. 229.24) and curcumin (CUR) (internal standard, IS purity >98% Mol. wt. 369.16) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Formic acid was bought from Sigma–Aldrich Co. (St. Louis, MO, USA). Formic acid and water were obtained from Avantor Performance Materials (Center Valley, PA, USA) and Fisher Scientific Korea Ltd (Seoul, Korea), respectively. Analytical grades of chemicals are used. A Millipore AFS-10 was used for purifying deionized water (Millipore, Billerica, MA, USA).

Preparation of RV loaded liposphere

The RV loaded liposphere is formulated by the solvent evaporation method (7). The required amounts of RV (0.02 g), capmul MCM C10 (0.2 g) and PL90G (0.8 g) were dissolved in chloroform and methanol (70:30 v/v). In addition, with a rotary film evaporator, the organic solution was steadily evaporated at 50–60°C with a reduced pressure. With a temperature of 50–60°C, the resulting solution was blended with 18.99 mL of water with continuous mixing until a uniform dispersion was received. The temperature was further decreased to 10°C and continuous rotation at 150 rpm. It was sonicated in a bath sonicator for 15 min, then refrigerated to 20°C and shaken again for another 5 min, contributing to homogenous dispersion of RV-containing liposphere.

Chromatographic and mass spectrometric conditions

Mass lynx mass lynx SCN918 (XEVO-TQD#QCA896) software version 1.50.2736 and firmware version 1.50.318 were used to perform analytical process development. The high quartz (MS1/MS2) analyzers with higher resolution and stability were used in this specific device. In comparison, the air vacuum turbo molecular pomp, which evacuated the source and analyzer with one rotary back pump had low noise, reliable photomultiplier detectors and single split flow. This is a collision cell known as T wave, which is triggered to maximize MS/MS output at high data acquisition speeds. The column contacts with the mobile phase to attain stable saturation, after that drug solution had been inserted into the chromatographic examination. ACQUITY UPLCr BEH C18 1.7 µm with serial number columns 01683913315522 and syringe size of 100 is used in chromatographic study. The column has been held at a temperature of 40°C and the autosampler maintained at 10°C. The injected sample containing 10 µL and elucidated the analytes by adding the mobile phase at a gradient elution in the ratio of methanol (A) and 2% formic acid (B) (ratio of 10: 90% v/v, 80: 20 v/v) and solvent system flow with the rate of 0.3 mL/min. Measurement was assessed at 306 nm at a separation period of 3 min. MasslynxTM Version 4.2 has been used for data collection, evaluate and storage of data. To identify compounds of significance MS/MS was run using the optimistic ESI and multiple reaction monitoring (MRM)/ES+ mode. As collision gas, argon was used at the pressure of 3.3×10^{-5} and capillary voltage kept at 2.98 kV. The cone voltage supply for RV and CUR (IS) is 47 and 30 V, respectively. In addition, the cone gas flow supply at 50 L/Hr, desolvation gas flow at the rate of 1,000 L/Hr and desolvation temperature maintained at 398°C. Furthermore, for RV and CUR, the source temperature was maintained at 148°C and collision energies provided 24 and 17 eV, respectively. MRM/ES+ mode to track transitions between precursors-products ions was used to identify analytes of 229.17 > 107.04 m/z (time scan is 3 min, retention time is 1.48) for RV and IS 369.16 > 176.938 m/z (time scan is 2.8 min, retention time is 1.11), respectively. MassLynxV4.2 methods were used to measure the molecular weight of the parent and their daughter's ion.

Development of standard samples and quality controls

RV and IS stock solutions were obtained individually by adding methanol to make 1 mg/mL and stored until further analysis. RV containing stock sample is diluted with the adding of mobile phase and make the calibration range in the 2.5–2,000 ng/mL. The stock solution was also preserved for storage at −80°C. The membrane filter containing 0.22 µm pore size has been utilized to purify the

sample and to make calibration plots for chromatographic analyses, whereas, with the inclusion of a mobile phase, the IS inventory solution was diluted to render the amount of 100 ng/mL.

Preparation and extraction of samples in the rat skin

In the process of dermatokinetic test, analytical system development was conducted in a rat skin to estimate RV. Further, the skin homogenate was used to extract and diluted up to the 2,000 ng/mL of RV-loaded liposphere and added to the centrifugal tube to make up the volume of 2 mL. A rat skin extract is used to make various dilutions up to (i.e. 2,000 ng/mL) loaded and added to the centrifugal tube by inserting a methanol (A) and 2% formic acid (B) mobile phase (10: 90% v/v, 80: 20% v/v) in the final volume. The cleansed skin was soaked in warm water (60°C) for 30 s to help separate the epidermis from dermis. All parts were individually sliced into tiny pieces and macerated for 24 h at the temperature of 35°C into a 10 mL methanol for full drug extraction. A 0.22 µm pore size of the membrane filter was used to filter supernatant and until study it preserved in cooled storage condition. The 10 µL aliquots are injected into the UPLC–MS/MS for linearity, consistency and precision (intra-day and inter-days) testing as according to section given below in order for testing.

Method of validation studies

This methodology was tested according to the United States Food and Drug Administration (USFDA) guidance on bioanalytical validity processes (8), which evaluated linearity, precision, recovery and system efficiencies.

Specificity, calibration, linearity and lower limit of quantification

No impurities were found during the retention time of RV and IS, during the studies that demonstrated the specificity of the technique. The linearity for the drug solution concentration range 2.5–2,000 ng. mL^{-1} was determined and make plotting the chart on the peak area against the concentration of drug solution range taken. Furthermore, the %RE and %CV requirements follow the approval limits in compliance with the validation of USFDA analytical studies. The lower limit of quantification (LLOQ) was 2.5 ng/mL of RV in rat skin.

Precision and accuracy

Precision and accuracies are estimated at four various drug concentration solutions named as (higher quality control (HQC), 2,000 ng. mL^{-1}; medium quality control (MQC), 1,000 ng. mL^{-1}; lower quality control (LQC), 20 ng. mL^{-1} and LLOQ, 2.5 ng. mL^{-1}) at various durations in a day (i.e. intra-day precision or repeatability) and various days (i.e. inter-day or intermediate precision). In addition, to verify the precision in the specified limits, the average percentage recovery (RE%) and percent relative standard deviation (%RSD) were estimated, whereas the appropriate limit for accuracy and precision, and the RSD value should be within ±15% and below 15%, respectively. In addition, the accuracy was calculated by means of the following Equation (1).

$$\text{Accuracy} = \left[(\text{measured concentration} - \text{nominal concentration}) / \text{nominal concentration}\right] \times 100 \quad (1)$$

Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were determined by applying standard deviation (SD) of the three-blank sample, injected solvents and spike (s) response of the calibration plot. After all, diluting the known concentration of RV to around 5 or 10 times, the standard variation of the response of the triplicate, the LOD and LOQ were determined. In addition, LOD and LOQ are calculated through the use of Equations (2) and (3).

$$\text{LOD} = \text{SD} \times 3.3/\text{Slope} \quad (2)$$

$$\text{LOQ} = \text{SD} \times 10/\text{Slope} \quad (3)$$

Recovery

The recovery from extraction was determined by contrasting the RV/IS ratios of quality control requirements of RV and derived with the same quality monitoring requirements extracted from homogeneous rat skin of IS. The RV from rat skin samples was retrieved with the application of LLE method.

Method application in dermatokinetic studies

Animal Study Protocol

The experimental protocols reviewed and adopted by the Regulations of the Institutional Animal Ethical Committee allowed in a Protocol number (Reg. No. 1840/PO/ReBi/S/15/CPCSEA) for control and monitoring of animal studies. The Wistar rat has been divided into two groups, each group contains six rats ($n = 6$), and their average weight is 160–200 grams. Both the groups of rats were acclimatized as regular free access to water and food; 25 ± 2°C, 12/12 h light and dark day. The circumstances and climate were closely watched for both the groups.

Ex vivo studies with Dermatokinetic modeling

Permeation tests were performed on male Wistar albino rats' excised abdominal skin (9). In short, cervical dislocation killed the animals. The fur was cut from the animal with surgical scissors, and the hair was removed with an operative blade number 24 from the secluded skin. The skin thickness was calculated using a Vernier caliper and the dye test checked for consistency of the skin. The cut skin rubbed out to eliminate excess fat and washed with ethanol: phosphate buffer (v/v) pH 6. The ready skin was covered in aluminum foil and placed for further use in a deep freezer of −30°C (10). To determine skin integrity by using methylene blue dye test (10), *ex vivo* drug-permeation studies were performed on an assembly of Franz diffusion cell, where the excised dorsal skin fixed between of donor and receiver compartment of diffusion cells (Perme Gear, Inc., Hellertown, PA, USA). The excised skin was transferred from the donor to the recipient portion, with an effective region of 2 cm^2 and a sink volume of 25.0 mL (10). As diffusion medium for preserving sink conditions, the receptor compartment includes absolute ethanol and the temperature has been preserved at 37 ± 0.5°C. The developed formulations RV loaded liposphere gel and RV suspension equivalent to 0.020% w/w of RV. Further, it applied on to the rat skin of the donor compartment. Periodic aliquots of an identical amount (1 mL) of fresh diffusion media were extracted from the sampling port at an acceptable time interval, in order to maintain the persistent volume of the receiver. Furthermore, the samples have been evaluated by using UPLC–MS/MS. The whole skin was stripped from Franz cell (0–5 h) and washed thrice to extract any sticky material in the dermatokinetic modeling experiment (10). In order to separate the epidermis from dermis, the cleansed skin was submerged for 30 s into warm water (60°C). All parts were individually sliced into tiny pieces and macerated for 24 h at the temperature of 35°C into a 10 mL methanol for full drug extraction. A 0.22 µm membrane filter was used to filter the supernatant. The filtrate has been analyzed

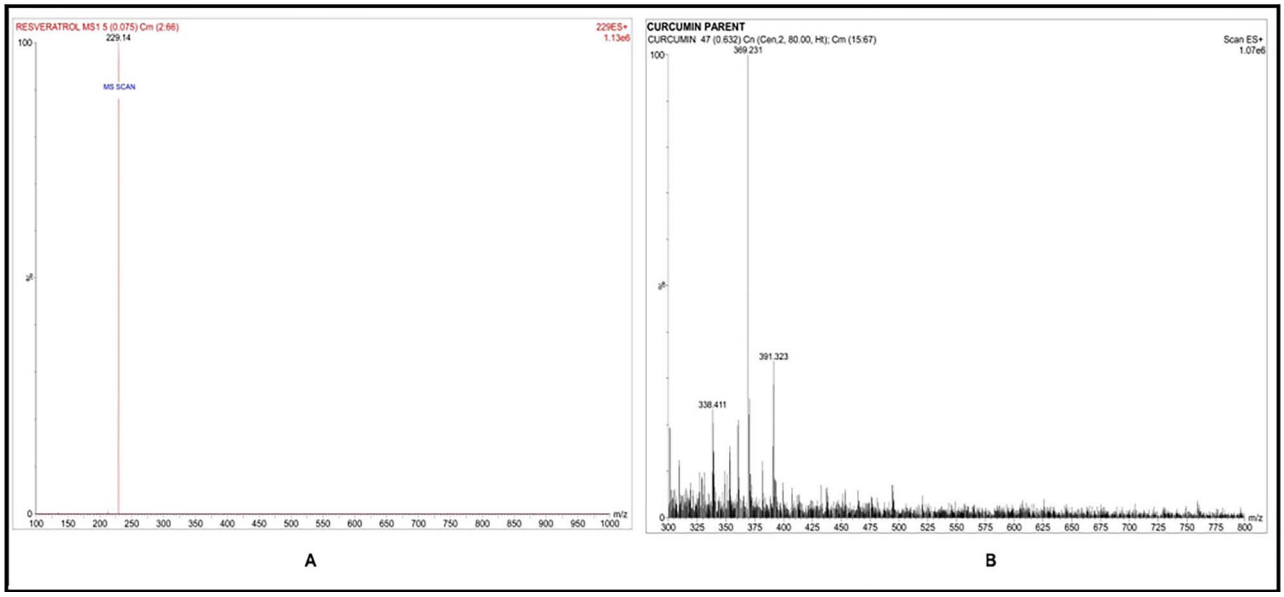

Figure 1. Represent the overall parent mass scan spectrum (A) RV (molecular weight 229.14) and (B) CUR (molecular weight 369. 231) as in the positive mode of ion.

using UPLC–MS/MS. The data obtained were built into a single compartment configuration according to Equation (4).

$$C_{Skin} = \frac{Kp.C_{max}^{Skin}}{(Kp - Ke)}\left(e^{-Kpt} - e^{-Ket}\right) \quad (4)$$

where C_{skin} is the RV available in the skin layers at time (t). Kp reflects the skin permeation constant, C_{max}^{Skin} indicated the maximal drug concentration available in the epidermis/dermis. K_e indicated that skin elimination constant. The various dermatokinetic parameters such as, C_{max}^{Skin} K_p, K_e, T_{max}^{Skin} (time required to achieve C_{max}^{Skin}) and area under the curve ($AUC_{0\text{-}12h}$) is analyzed by using Win-Nonlin Ver 5.0 software by employing Wagner-Nelson method (10).

Statistical Analysis

On average ± SSD of the results is shown. In addition, *t*-test students are being used for evaluating the pharmacokinetic findings. The variations are taken into account at the standard of $P < 0.05$.

Results and discussion

Selection of IS

CUR is the cornerstone to bioanalytical science and is a satisfactory IS. The studies prove CUR to be used as an IS because of its utility, stability, quality of extraction and precision of the study during the retention time (9). To apply in the analytical approach development and evaluate their dermatokinetics, CUR has been chosen as IS for its related chromatographic action to the RV. CUR was found to be feasible and satisfactory as IS because of a successful adjustment with RV.

Spectrometric and chromatographic conditions and their optimization

The parent's ion $[M+H]^+$ of 229.14 and 369.231 m/z, each showed the full-scanned mass spectrum of RV and IS received by quantitative approach. (Figure 1A and B). The optimum collision energy, cone voltage is employed 40 eV, 60 V, 12 eV and 28 V to produce the daughter ions 107.17 m/z and 176.938 m/z for RV and IS, respectively (Figure 2A and B). RV and IS in positive ESI MRM mode were observed. The solvent system has established a stable and sufficient peak for RV and IS with 2% formic acid (B) and (10: 90% v/v, 80: 20 v/v) methanol (A). The retention time was found 1.48 and 1.11 min, respectively, after optimization. Further, the chromatogram of RV and CUR solution shows in Figure 3A–B.

Calibration plot and linearity range

Figure 4 presents a linear calibration plot of RV although, in addition, linearity estimation is taken into account in reference to a percentage bias of ±5%. Moreover, no one point was observed externally and suggested linearity over the range (9, 11, 12). The findings suggest a linearity for the concentration of the drug between 2.5 ng/mL and 2,000 ng/mL ($R^2 = 0.987$). The system is established and verified its efficacy through regular practice of the analytical process.

Precision and Accuracy

RV consistency outcomes are measured using four different quality control samples (intra-day and intra-day) as shown in Table I. All the results (precision and accuracy) of the tested samples from the rat skin were within the acceptance limit (RE%: ±15; RSD%:15). These results have shown that the system established is highly reliable and was based on the USFDA guidance on the validation of the bioanalytical process. Table I also provides accuracy results for RV from various quality control samples (RV).

Recovery

The results for recovery studies are shown in Table II. Overall, RV mentioned as HQC (2,000 ng. mL^{-1}), MQC (1,000 ng. mL^{-1}), LQC 20 ng. mL^{-1}), LLOQ (2.5 ng. mL^{-1}) showed a good per-

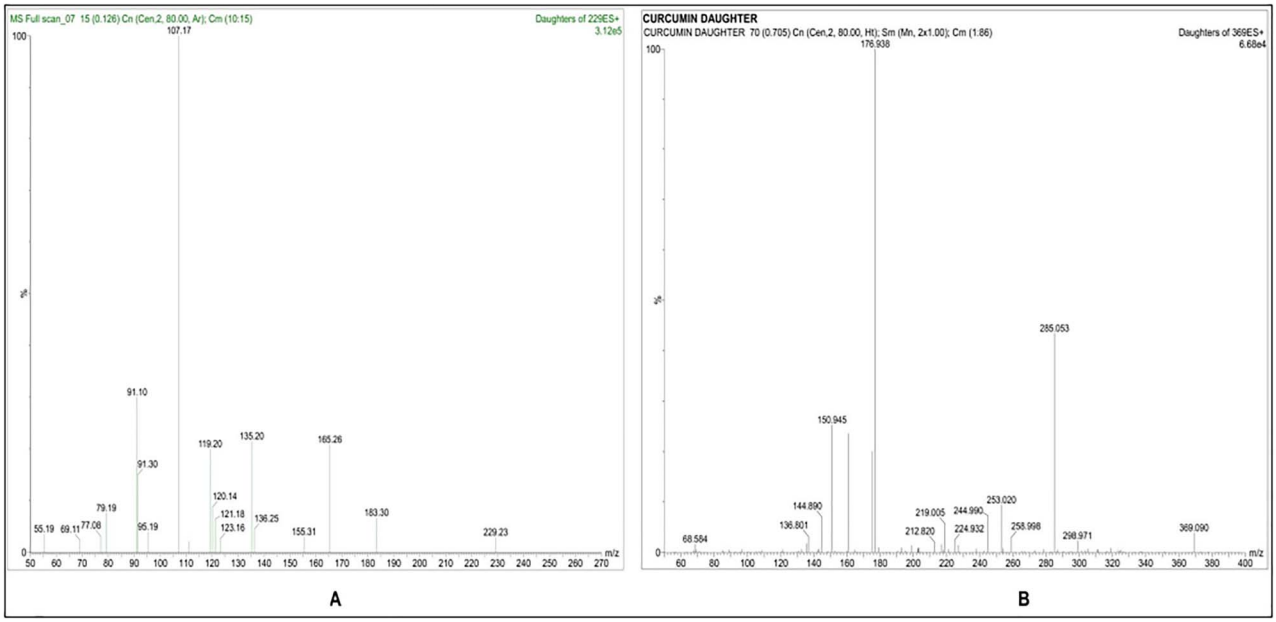

Figure 2. The daughter ion [M + H] + ion spectra are illustrated by (A) RV (molecular weight 107.17) and (B) CUR (molecular weight 176.93) as in the positive ion mode.

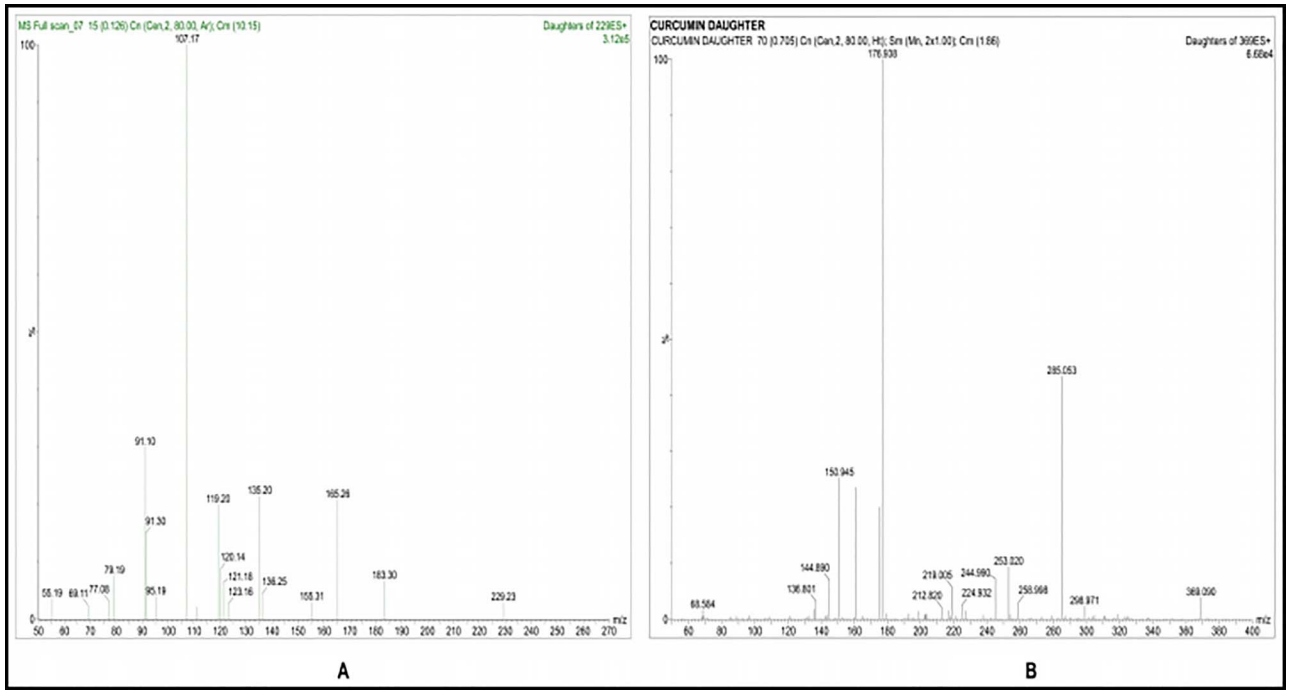

Figure 3. Chromatograms achieved following MRM of (A) RV as pure drug and (B) CUR as IS.

centage recovery and found within acceptance limit (90–110%). These observations showed specifically that co-eluting matrix and impurity did not impact RV and IS ionization. Extraction recovery was determined by the contrast of the RV/IS peak ratios of quality control standards without extraction have the same quality control standards, which were derived from the skin homogeneity. The LLE approach supported good recovery of RV from skin samples.

LOD and LOQ

It was observed that LOD and LOQ values are 2.5 and 20 ng. mL^{-1}, respectively. The method has described the framework for the quantification of RV has a reasonably high sensitivity.

UPLC–MS/MS method development in rat skin

RV bioanalytical method in rat skin was based on the above findings. Figure 5A, displays the chromatogram of the blank peak into the skin

Table I. Precision and accuracy of RV in Skin Homogenate of rat ($n = 6$)

Matrix	$C_{nominal\ (ng/mL)}$	Intra-day			Inter-day		
		$C_{measured}$ (ng/mL)	RSD (%)	Accuracy (%)	$C_{measured}$ (ng/mL)	RSD (%)	Accuracy (%)
Skin	2,000 (HQC)	2,022.1 ± 2.11	2.9	2.3	2,021 ± 2.90	2.8	2.2
	1,000 (MQC)	1,003.1 ± 5.21	2.5	2.1	1,001 ± 4.41	2.7	1.4
	20 (LQC)	20.3 ± 6.31	2.8	1.3	20.1 ± 5.31	2.9	1.2
	2.5 (LLOQ)	2.3 ± 3.21	3.5	1.4	2.2 ± 3.1	4.0	−1.1

QC samples (HQC-High, MQC-Medium, LQC-Low, LLOQ- Lower limit of quantification).

Table II. Recovery (RE) of RV and IS in the Skin ($n = 6$)

Analyte	$C_{nominal}$ (ng/mL)	Recovery (%)
RV	2,000 (HQC)	91 ± 2.4
	1,000 (MQC)	92 ± 3.1
	20 (LQC)	90 ± 4.5
	2.5 (LLOQ)	88 ± 4.2
CUR (IS)	100	85 ± 3.1

Table III. Dermatokinetic Parameters of RV Loaded Liposphere Gel in: (A) Epidermis and (B) Dermis at Various Time Intervals

Dermatokinetic parameters	RV-Liposphere gel		RV suspension	
	Epidermis	Dermis	Epidermis	Dermis
$AUC_{0\text{-}24h}$ (μg cm^{-2} h)	1,716.55 ± 1.75	1,945.621 ± 1.21	782.11 ± 20.12	602.82. ± 1.11
C_{max} (μg cm^{-2})	80 ± 3.7	60 ± 1.11	45 ± 2.6	30.01 ± 5.12
T_{max} (h)	1 ± 1.02	1.0 ± 1.38	1 ± 1.07	1.0 ± 0.45
$T_{1/2}$	41.02 ± 1.99	61.083 ± 21.08	22.117 ± 2.02	34.698 ± 21.04
MRT	55.157 ± 21.48	83.918 ± 1.03	30.293 ± 1.21	47.161 ± 31.4

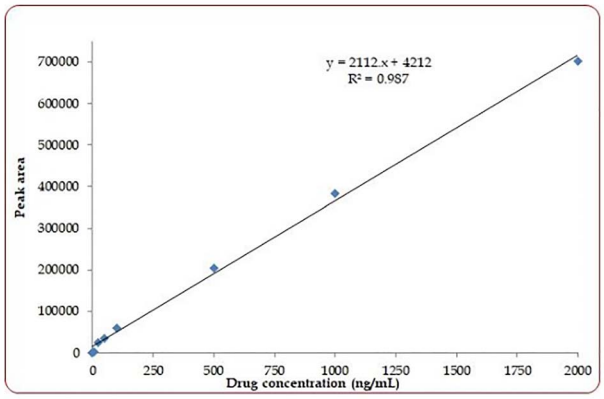

Figure 4. Linear calibration plot of RV in the mobile phase.

and shows no peak appearance over time. The chromatograms for RV and CUR solutions demonstrated the same symmetry and no variation found in rat skin samples (Figure 3A and B) as well as further RV loaded liposphere gel and CUR solution (Figure 5B and C).

Calibration plot and validation of linearity range

RV calibration curves were found linear over the range of 2.5–2,000 ng. mL^{-1}. In comparison, linear R^2 provided the least square regression analysis 0.984. This indicates that the proportion of biases from all reactions was below ±5% and none of the points found exceeding outlines indicated a high degree of similarity with the concentrations expected (8, 9, 11).

Precision, accuracy and recovery

Four different concentrations such as 2.5, 20, 1,000 and 2,000 ng.mL^{-1} of the drug showed all the results (precision and accuracy) of the tested samples from the rat skin also were within the acceptance limit (RE%: ±15; RSD%:15). Therefore, the developed methods are extremely reliable and validated for bioanalytical method according to USFDA guidelines (8). Although the recovery of RV in skin samples for all above aforementioned samples is showed a good percentage recovery and found within the acceptance limit (90–110%).

Method application to ex vivo permeation studies

The permeation results observed better drug transport observed with the RV liposphere gel to the skin over the RV suspension. Because of their strong permeations and retention effects in skin layers, this can be related to the dominance of liposphere gel carriers by the incorporation of carrying components into skin lipids. The distribution of the drug to the Wistar male rat skin is illustrated in Figure 6A and B. RV was found to be significantly higher in skin layers with liposphere gel ($P < 0.05$) compared to the RV suspension. Table III revealed the AUC_{0-24h}, C_{max}^{Skin}, T_{max}^{Skin} and $T_{1/2}$ and mean residence time (MRT).

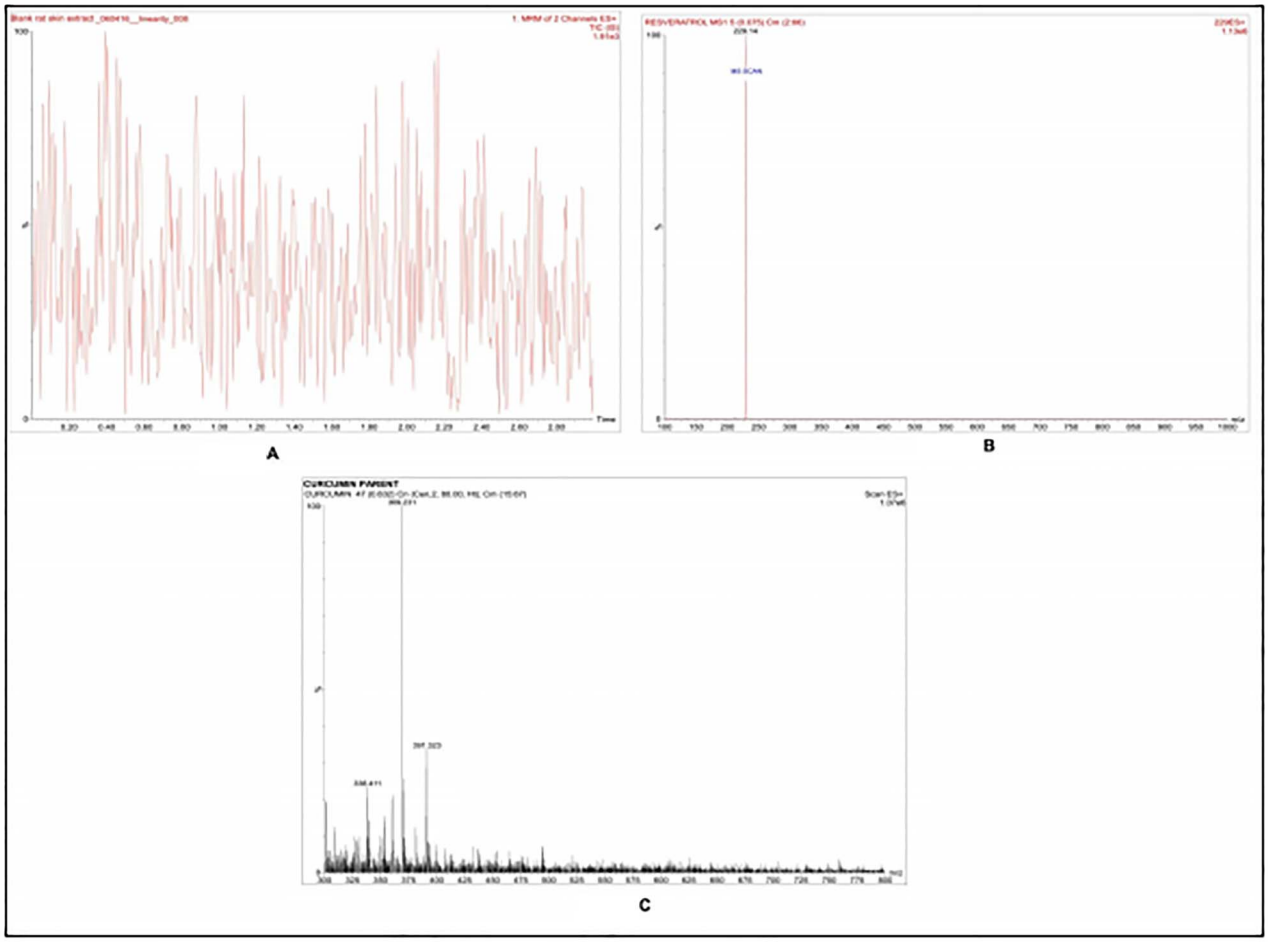

Figure 5. Chromatograms of RV and CUR as IS detected on the rat skin following MRM; (A) Blank rat skin extract, (B and C) typical chromatograms of rat skin sample collected at 12 h following treatment with RV loaded lipospheres gel and CUR loaded lipospheres gel.

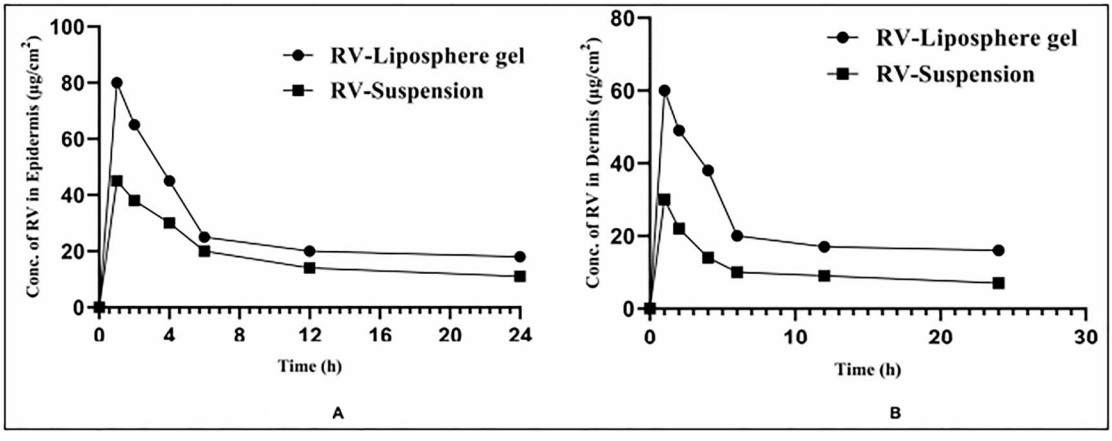

Figure 6. Dermatokinetic profile indicating the concentration of the RV at different points in: (A) epidermis and (B) dermis.

Results show that RV-liposphere gel in both the skin layers has improved the therapeutic half-life of RV and drastically decreased T_{max}. C_{max} was significantly improved in both layers and AUC in the dermis. The findings have explicitly confirmed that the liposphere gel is capable of increasing RV delivery and increasing the topical bioavailability of conventional products.

Conclusions

The linearity, specificity, precision and recovery found according to USFDA guidelines and the development of a UPLC–MS/MS system for quantitative RV measurement in the rat skin have been validated. In addition, this approach has expanded its use to successfully evalu-

ate the quantification of RV from RV-loaded liposphere gel in rat skin. Moreover, easier extraction, less solvent usage and shorter retention duration allow this analytical approach for RV quantification to be used in a broad range of topical dosage forms.

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