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A VALIDATED RP-HPLC-UV-VISIBLE METHOD FOR QUANTIFICATION OF SWERTIAMARIN USING SPE METHOD AND ITS APPLICATION TO PHARMACOKINETIC STUDY USING SPARSE SAMPLING METHODOLOGY

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ABSTRACT

Swertiamarin (SWM), a secoiridoid glucoside from *Swertia densifolia* (Griseb.) Kashyap, has shown biological activities like antidiabetic, antihelmenthic, antinociceptive, hepatoprotective, antioxidant, etc. The present paper describes a selective and sensitive Reverse Phase – High Performance Liquid Chromatography method (RP-HPLC) using fixed wavelength UV detector for quantification of SWM in rat plasma. Briefly, the plasma samples were pre-treated using a simple solid-phase extraction method. The separation of SWM and the internal standard, Hydrochlorothiazide (HCTZ), was achieved on Cosmosil C18 column (150 mm X 4.6 mm i. d., 5 µ) using 1.0 mL/min isocratic mobile phase flow. Also, the developed bioanalytical method was validated as per the US FDA bioanalytical guidelines over the concentration range of 50.0 to 40,000.0 ng/mL (r^2 > 0.9980) for SWM from rat plasma. The mean percentage recovery (n=6) for the low, middle and high quality control samples was 91.23 ± 1.88, 90.36 ± 2.69 and 87.52 ± 1.50 %, respectively. The applicability of the validated RP-HPLC method was demonstrated by successful measurement of SWM from plasma following oral administration of *Swertia densiflora* (Griseb.) Kashyap leaf extracts to female Albino Wistar rats.

KEYWORDS: Swertiamarin, RP-HPLC, Method validation, Pharmacokinetics, *Swertia densifolia* (Griscb.) Kashyap

INTRODUCTION

Swertiamarin (SWM), carboxylic acid ester, is a secoiridoid glucoside, an active biomarker found in members of Gentianaceae family like Swertia chirata (Wall) Clarke, Swertia ciliata, Swertia japonica Makino, Swertia angustifolia Butch.-Ham.ex D.Don and Swertia densifolia (Griscb.) Kashyap. Swertia densifolia is an annual herb which is also known as Swertia decussate Nimo.ex Grah. is a native of Northern Deccan and Mahabaleshwar region of Penninsular India. The plant is well known potential substitute of Swertia chirata (Wall) Clarke, which is an important medicinal plant used in many Ayurvedic formulations in India. The plant is a bitter stomachic tonic, febrifuge, laxative and also prescribed as blood purifier in skin diseases (Shailajan, et.al. 2009).

SWM content is reported to be found maximum in the month of March when plant is in flowering and seed bearing condition (Shailajan, et.al. 2009). Hence, the test plant material was collected during this period. SWM, (4aR,5R,6S)-4a-Hydroxy-1-oxo-5-vinyl-4,4a,5,6-tetrahydro-1H,3H-pyrano[3,4-c]pyran-6-yl β-D-glucopyranoside, reports bioactive activities like antidiabetic, antinociceptive, hepatoprotective, antihyperlipidaemic, antioxidant, etc. (Patel, T., *et.al*., 2013, Vaidya, H., *et.al*.,2009a, Jaishree, V., *et.al*., 2009, Jaishree, V. and Badami, S., 2010). Therefore, to support the preclinical and clinical findings in order to achieve a better understanding of the pharmacological effects of SWM, development of a reliable bioanalytical method was essential.

There are numerous methods reported to detect SWM from raw plant materials as well as marketed formulations using Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) (Ahmad, J., *et.al.*, 2014, Alam, P., *et.al.*, 2009, Rana, V.S., *et.al.*, 2012,), Capillary Electrophoresis (CE) (Takei, H. *et.al*., 2001) and Liquid-Chromatography-tandem Mass Spectrometry (LC-MS/MS) (Suryavanshi, S., et.al. 2007, Aberham, A., *et.al*., 2007, Suryavanshi, S., *et.al*. 2006) . Although, various studies have reported for pharmacological action of SWM in pigeons, rodents and dogs in past years, limited assays are reported for quantification of SWM in biological fluids and tissues from liver and kidney (Mandal, S., 1998, Li, H.L., *et.al*., 2012, Gui-li, Xu, *et., al*., 2013, Hong-Liang, Li, *et., al*., 2011). Some of the drawbacks of the reported methods to quantitate SWM from plasma are low sensitivity of the method with linear range of 5.0-1000.0 ng/mL and use of organic solvent in higher ratio

in the mobile phase as compared to current method. Also, the methods reported for detection and quantification of SWM in raw plant materials and marketed formulations were found difficult to be applied to quantify SWM from the biological matrix i.e. rat plasma. Since, current method is developed for RP-HPLC instrument its ruggedness increases as compared to previously reported LC-MS/MS methods. Current method can be applied to pharmacokinetic studies with higher dose of SWM due to its broad range of concentrations used in linearity.

The objectives of current research work were; (a) to develop a sensitive and selective RP-HPLC method for determination of SWM from rat plasma using an internal standard (b) to validate the developed method as per US FDA guidelines; and (c) to demonstrate the applicability of the method to pharmacokinetic study in rats after administration of hydroalcoholic extract of *Swertia densifolia* leaves.

MATERIALS AND METHODS

Plant Material and extract preparation

The plant material was collected from Mahabaleshwar, Dist. Satara, Maharashtra, India, in the month of March when SWM contents in the plant are highest (Shailajan, S., *et.al.*, 2009) and was identified as *Swertia densifolia* (Griseb.) Kashyap, by the experts from Botanical Survey of India, Pune. The leaves were washed with the water and air dried. Foreign organic matter was removed. The cleaned plant material was shade dried for 4-5 days and then kept in hot air oven at 37 ± 2°C for 48 hours. The dried plant material was powdered and sieved through BSS mesh no. 85 to obtain the uniformity in the test plant material. The sieved plant material was stored in polypropylene container till further use.

The powdered sample (1.0 g) was extracted with 10 mL of 70% ethanol for 18 hours. The extract was then filtered with Whatmann filter paper no.1 and was evaporated to dryness in a centrivap vacuum concentrator system (Labconco Corp., MO, USA) to obtain leaf extract. The hydroalcholic leaf extract of *Swertia densifolia* (LESD) showed the yield of SWM as 1.35 mg/g by developed RP-HPLC method.

Chemicals and Reagents

The organic solvents and chemicals used for extraction under study are of analytical grade and procured from Qualigens Fine Chemicals, Mumbai, India. HPLC grade formic acid (98 - 100 %), acetonitrile, methanol and distilled water were procured from Merck, Mumbai, India.

Standard Swertiamarin (98% purity) was procured from Chengdu Biopurify Phytochemicals Ltd., China and Standard Hydrochlorothiazide (HCTZ) (98.5 % purity) was received as generous gift from Cipla Ltd., India. The representative chemical structures of SWM and HCTZ are shown in **Fig. 01 A and Fig. 01 B** respectively.

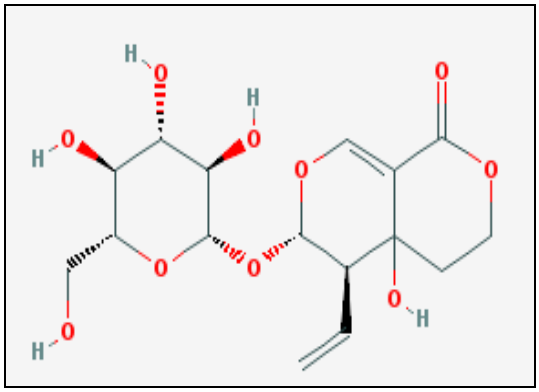

Fig. 01 A. Structure of Swertiamarin

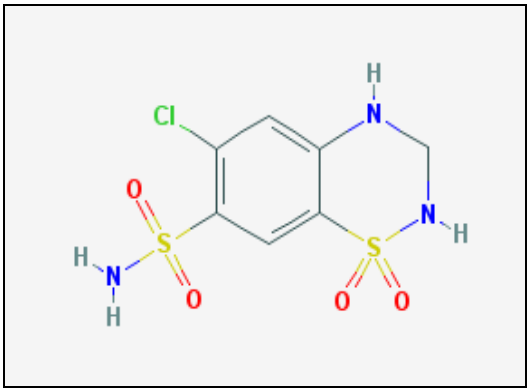

Fig. 01 B. Structure of Hydrochlorothiazide

RP-HPLC and MS/MS instrumentation

In RP-HPLC system the mobile phase used was 10 mM Ammonium acetate: Acetonitrile: Formic acid in the ratio of 85:15:0.2% (*v/v/v*). Flushing solvent used for the experiment was mobile phase. UV detector, Jasco UV-970, was used at a wavelength of 237 nm. Maximum pump pressure of 500 kg/cm^2 was set at Jasco PU-980 pump. Autosampler, Jasco AS-1527, was set at 4°C ± 2°C. Flow rate of 1.0 mL/min along with the injection volume as 50 µL were set as the chromatographic conditions. Cosmosil C_{18} (4.6 mm i.d. x 150 mm, 5 µ particle size) was used as column. Data acquisition and analysis were performed using Borwin software version 1.50.

Mass analysis was performed using Tripple - quadrupole Tandem Mass spectrometer, an API Applied Biosystems hybrid Q-TRAP 2000 Mass Spectrometer (AB-MDS Sciex, Toronto, Canada) equipped with electrospray ionization source (ESI). For data acquisition and analysis Analyst software 1.3 (AB Sciex, USA) was used with Multiple Reaction Monitoring. ESI was operated on positive ionization mode. Sample solutions of 1 ppm SWM and hydroalcoholic extract of test plant material (1:10,000 dilution) diluted using 10 mM ammonium acetate : methanol in 1:1 ratio were infused at 10 µL/min using syringe pump method. The MS parameters optimized were as follows: Curtain gas (CUR), Ionization potential (IP), Nebulizer gas (GS1) and Heater gas (GS2) were 20 psi, 3500 eV, 60 psi and 0 psi respectively. The CAD gas was set to medium while the ion source temperature was

maintained at 350°C. The quadrupole Q1 and Q3 functioned in the unit resolution mode with a dwell time of 299 ms for every MRM transition.

Heavy Metal Analysis

The plant was evaluated for heavy metals like cadmium, arsenic, mercury and lead. Heavy metal analysis in raw plant material is important as plants can be easily get contaminated by heavy metals during growth, development and processing (Agoramoorhty, 2007; Bandaranayake, 2006; Shailajan *et al*, 2004). The samples were analyzed externally at Shradhdha Analytical Services, Mumbai, India. Inductively coupled plasma/optical emission spectrometry (ICP/OES) by Perkin-Elmer, a powerful tool for the determination of metals in a variety of different sample matrices was used for the evaluation at Shradhdha Analytical Services.

The procedure followed for sample processing is as follows; 0.2 g of test plant material was digested with 10 mL of conc. Nitric acid on hot plate for 30 mins at 200°C. Then the sample was allowed to cool down. Then 10 mL of distilled water was added and was further diluted upto the mark in 25 mL standard volumetric flask. The standards used for comparison for arsenic, cadmium and lead were of 0.5 ppm, 1.0 ppm, 2.0 ppm concentration and for mercury standards used were 0.05 ppm, 0.1 ppm and 0.2 ppm.

Preparation of calibrator and quality control (QC) samples

Two sets of SWM and HCTZ, stock solutions of concentration 1.0 mg/mL were prepared by independent weighings in methanol. One set of SWM was used to prepare calibrant working solutions while the other set to prepare quality control working solutions in diluent. The diluent used was acetonitrile:10 mM ammonium acetate: formic acid (15:85:0.2 %, *v/v/v*). All the stock and working solutions were stored at 2–8 °C and were brought to room temperature before use.

An eight point standard calibration curve for SWM at concentration levels of 50.0, 100.0, 500.0, 1000.0, 5000.0, 10,000.0, 20,000.0 and 40,000 ng/mL was prepared by spiking blank rat plasma 240 µL with respective calibrator working solutions (10µL). Similarly, control samples at concentration levels 400.0, 7500.0 and 30,000 ng/mL for SWM were prepared in rat plasma and designated as low, middle and high quality control samples, respectively.

Sample Processing and Preparation

Pharmacokinetic (PK) study samples stored at -70°C were thawed at room temperature for processing. The samples were brought to uniformity by vortex mixing of samples for 30 seconds. Then, 250 µL of the rat plasma sample was transferred to polypropylene tubes. Subsequently, samples were spiked with 10 µL of 100 ppm of HCTZ working solution. 25 µL of concentrated ortho-phosphoric acid was added followed by 0.5 mL of acetonitrile to the samples and were vortex mixed for 30 seconds. The samples were introduced to previously conditioned DVB-LP SPE cartridges. Conditioning of cartridges was performed by 2 mL methanol and 2 mL 10 mM ammonium acetate. The loaded samples were flushed with 2 mL 0.1 % formic acid followed by final elution of SWM and HCTZ with 2 mL methanol. This eluted methanol was then evaporated to dryness using centrivap vacuum concentrator system (Labconco Corp., MO, USA) at 60°C. The residue was then reconstituted with 125 µL of mobile phase. After vortex mixing for 30 seconds, 50 µL of sample was injected in RP-HPLC system for analysis.

BIOANALYTICAL METHOD VALIDATION

The developed RP-HPLC method for determination of SWM from rat plasma thoroughly validated in accordance with the US FDA guideline for bioanalytical validation (US DHHS, *et., al*., 2001).

System Suitability

System suitability tests are integral part of liquid chromatographic methods. The test was performed to verify that whether system was adequate for the intended analysis. Replicate injections of the standard solution of SWM 1 µg/mL in combination with standard solution of HCTZ (n = 6), were compared to ascertain whether requirement for precision meet. The data generated by these injections was used to calculate % relative standard deviation.

Linearity (Calibration Curve)

Four calibration curves consisting of 8 point concentration levels of range 50.0-40,000 in ng/mL of SWM in rat plasma were prepared and analyzed on four consecutive days. The calibration curves were a plot of peak area ratios of SWM and IS vs the concentration of SWM constructed on the basis of weighted linear regression ($1/ x^2$). Deviations of the back-calculated concentrations for the calibration standards were set at ±15 %, whereas for Lower Limit of Quantification (LLOQ) it was ± 20 %.

Selectivity and Sensitivity

Blank plasma samples obtained from six different rats were processed and analyzed in order to investigate the potential interferences from endogenous substances. The chromatograms obtained for blank, zero standard (spiked with IS only), non-zero standard (spiked with analyte and IS) and an in vivo study sample were compared to ascertain the selectivity of the method.

The limit of detection (LOD) and limit of quantitation (LOQ) for the developed method was determined by injecting progressively low concentrations of the standard solution of SWM. Limit of detection (LOD = 25 ng/mL) and limit of quantitation (LOQ = 50 ng/mL) were established at a signal to noise ratio of 3:1 and 10:1 respectively.

Precision and Accuracy

The precision and accuracy (P & A) of the system was determined by measuring repeatability of sample application and measurement of concentration for six replicates at three different concentrations of 400.0, 7500.0, 30,000.0 ng/mL. Intra and inter-day variation for the determination of SWM were carried out. The intraday precision was carried out on the same day while inter-day precision (intermediate precision) was studied by comparing assays performed on three different days. The precision of the system and method were expressed as percent Relative Standard Deviation (% R.S.D.) and % Relative error (% R.E.). The acceptance criteria for the intra-day and inter-day precision and accuracy are within ±15% for all levels.

Dilution Integrity

A dilution integrity experiment was performed with the aim of validating the method to be carried out for the samples with concentrations beyond the linear range during *in vivo* sample analysis. Three replicates at 2-fold and 4-fold dilutions were prepared by giving appropriate dilutions to the two times highest level of the calibration curve with blank plasma and their concentrations calculated by applying the dilution factor.

Ruggedness

Ruggedness was determined by change in instrument, column and analyst of assay procedure (Ahamad, et.,al., 2014). To authenticate the ruggedness of the proposed method, it was done on two precision and accuracy batches. The first batch was analyzed by different analysts

while the second batch was analyzed on different column and different RP-HPLC-UV-Visible system.

Stability

Stock solution stability of SWM was assessed by evaluation of short-term stability for 24 h and long-term stability for up to 30 days. The area responses for the stability solutions were compared with that of the freshly prepared solutions with an acceptable deviation of ± 10% (D.C. Reddy et.,al., 2012). The stability of SWM in rat plasma was ascertained by analyzing in six replicates the quality control samples kept under following storage conditions: bench-top stability for 4 hrs; process stability for 24 h at 8°C; long-term stability was evaluated by extracting the samples kept at -70 ± 5°C for 30 days; freeze-thaw stability over five cycles; wet extract and dry extract. These stability samples were compared with freshly extracted quality control samples. The percentage difference between the stability and freshly prepared samples was of ± 15 % was considered acceptable (D.C. Reddy et.,al., 2012).

Recovery and Process efficiency

The extraction recovery and process efficiency were evaluated by analyzing the quality control levels according to the methods described by Koo, *et.al*. The extraction recovery of SWM and IS were determined by peak areas of analyte/IS spiked before extraction into plasma with peak areas for analyte/IS spiked after extraction. The mean process efficiency was evaluated by comparing peak areas of analyte/IS spiked before extraction into blank plasma with peak areas of analyte/IS for neat solution of analyte/IS prepared in reconstitution solvent.

Application to Pharmacokinetic study in Rats

The guidelines of Committee for purpose of Control and Supervision of Experiments on Animals (CPCSEA), government of India, were followed to prepare the study protocol. Institutional Animal Ethics Committee of Ramnarain Ruia College, (Mumbai, India) approved the study protocol after review of the protocol by the committee. 42 healthy female Albino Wistar rats (36 study animals and 20 % extra rats) of weight range 250.7 ± 10.78 g were obtained from Haffkine's Institute (Mumbai, India). The rats were housed in polypropylene cages and kept in a well-ventilated room of the animal house facility (CPCSEA Reg. No. 315; Ramnarain Ruia College, Mumbai India) under regulated temperature (22 ± 2° C), relative humidity (60 ± 5 %) and 12/12 h dark/light cycle. The rats were acclimatized for 1 week before the beginning of the study and daily body weight

monitored, in order to assess their general health. Standard pellet diet (Amrut Laboratory, Maharashtra, India) and water were provided *ad libitum*. For the pharmacokinetic study, the rats were fasted for 12 h prior to dose administration and 3 h post-dosing, after which food and water were given *ad libitum*. LESD (equivalent to 3 mg/kg of SWM) suspended in 4 mL of distilled water was administered orally to each rat. A sparse sampling methodology was employed for collection of blood samples from the dosed rats (i.e. three sampling points per rat, six samples per sampling point) (33, 34). In brief, 36 rats were divided equally into six groups (six animals in each group). Each group rat accounted for only three samples including the pre-dose sample (0.00 h), i.e. Group I rats for 0.00, 0.16, 1.75 h; Group II rats for 0.00, 0.33, 2.00 h; Group III rats for 0.00, 0.66, 4.00 h; Group IV for 0.00, 1.00, 8.00 h; Group V for 0.00, 1.25, 10.00 h; Group VI for 0.00, 1.50, 12.00 h. Blood samples of about 0.50 mL per sample were collected from the retro-orbital plexus of the rats under light ether anesthesia using heparinized capillaries into tubes containing potassium-EDTA (K3-EDTA) as anti-coagulant. After collection, blood samples were immediately centrifuged at 4000 rpm at 10 mins. The supernatant plasma was transferred into sample vials and stored at -70° C until further analysis. In addition, blank plasma samples from untreated rats for method development and validation were obtained from the animal house facility.

Data Analysis

WinNonlin computer software (version 3.1, Pharsight Corporation, Mountain View, CA, USA) was used to evaluate the pharmacokinetic parameters. The maximum drug concentration (C_{max}) and the time to reach the maximum concentration (T_{max}) were determined from the individual plasma concentration time profiles for each rat. A non-compartmental model was used to calculate AUC_{0-12} h (Area under the plasma concentration-time curve) by the log-linear trapezoidal method, extrapolated to infinity to obtain the last measurable plasma drug concentration ($AUC_{0-\infty}$). The terminal elimination rate constant (K_{el}) was obtained by linear regression of the final log part of the plasma concentration-time curve. The apparent elimination half-life ($t_{1/2}$) was also calculated based on the terminal concentration-time points. All results were expressed as the arithmetic mean ± standard deviation (Mean ± S.D.).

RESULTS AND DISCUSSIONS

Mass spectrometry in detection and optimization of SWM

Signal intensities obtained by the infusion pump method for SWM (0.001 mg/mL) were evaluated by monitoring the full-scan mass spectra in negative ionization mode. The mass

fragmentation pattern of SWM represented base peak m/z of 375 representing molecular weight and m/z of 212 (M-162) a characteristic peak after removal of sugar moiety from the compound (**Fig. 02 A** and **Fig.02 B**).

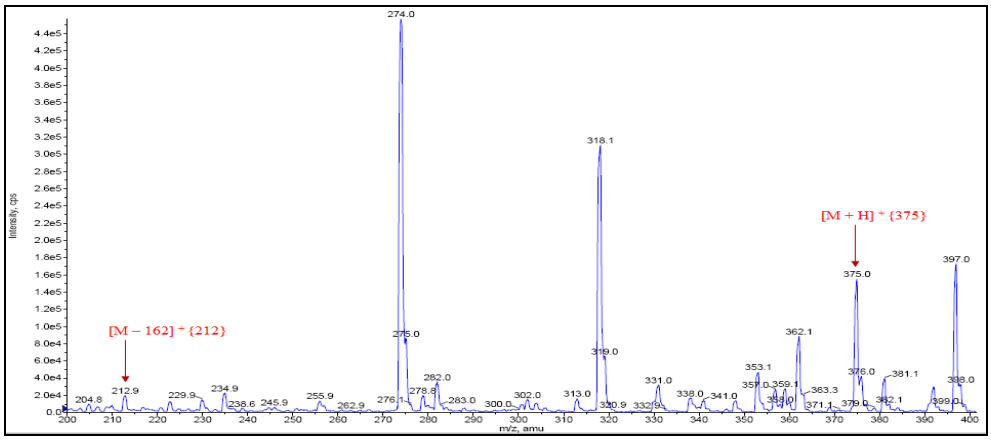

Fig. 02A MS spectra of Swertiamarin standard

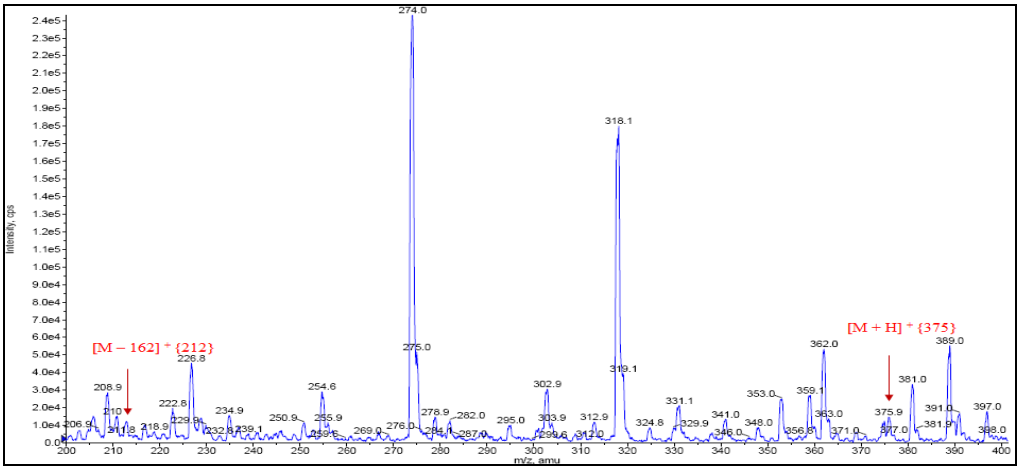

Fig. 02B MS spectra of *Swertia densifolia* leaf extract

Leaf extracts of *Swertia densifolia*, were prepared in different solvent and mixture of solvents. After pushing all the samples in MS/MS one by one by syringe infusion method and by comparing with 1 ppm standard it was found that 50 % methanolic extract, choloroform extract, n- butanol extract and 70% ethanolic extract of test plant material showed the presence of SWM. Hence, these extracts were used to calculate SWM contents in them by comparing with standard SWM of 1 ppm concentration using RP-HPLC method. It was found that 70 % ethanolic extract showed maximum yield of SWM as 1.35 mg/g.

Optimization of RP-HPLC conditions

There are numerous methods reported to detect SWM from raw materials as well as marketed formulations using Reverse Phase-High Performance Liquid Chromatography (RP-HPLC)

(Ahmad, J., *et.al.*, 2014, Alam, P., *et.al.*, 2009, Rana, V.S., *et.al.*, 2012,), Capillary Electrophoresis (CE) (Takei, H. et.al., 2001) and Liquid-Chromatography-tandem Mass Spectrometry (LC-MS/MS) (Suryavanshi, S., et.al. 2007, Aberham, A., et.al., 2007, Suryavanshi, S., et.al. 2006) . Various studies have reported for pharmacological action of SWM in pigeons, rodents and dogs in past years, limited assays are reported for quantification of SWM in biological fluids and tissues from liver and kidney (Mandal, S., 1998, Li, H.L., et., al., 2012, Gui-li, Xu, et.,al., 2013, Hong-Liang, Li, et., al., 2011). However, there is no RP-HPLC-UV-Visible method reported for SWM detection from rat plasma.

Some of the drawbacks of the earlier reported methods are low sensitivity of the method with LDR 5.0-1000.0 ng/mL. Current method is developed for RP-HPLC instrument is rugged and can be applied to higher dose of SWM as compared to the reported methods due to its large scale of LDR which is 50.0 to 40,000 ng/mL.

A comparison of C18 and C8 columns with different specifications was performed. It was observed that good separation, better resolution, sensitivity and selectivity were accomplished on a C18 column to which the binding of analyte is stronger. Further, it was seen that the type and concentration of organic modifier (acetonitrile or methanol), the formic acid concentration in the mobile phase and the buffer concentration affected the chromatographic separation. Acetonitrile on comparison with methanol gave a significant increase in signal intensity of SWM as well as HCTZ. A wide range of concentrations of formic acid (0.1 % to 1.0 %) was investigated. It was observed that peak response of SWM increased considerably when the concentration of formic acid in the phase was 0.2 %. Several different concentrations of ammonium acetate and ammonium formate (2–10 mM) buffer solution were tested. It was found that 10 mM ammonium acetate resulted in a good response and improved the peak shape for the analyte and IS. If the ratio of organic modifier is increased the analyte and IS peaks merge together and separation is very poor.

Therefore, the ratio of the mobile phase was finalized as 85:15:0.2% (*v/v/v*) that of 10 mM Ammonium acetate : acetonitrile : formic acid. Also the run length of the method was increased upto 10 mins which resulted in good resolution of the analyte from endogenous peaks from the test samples. Under the optimized chromatographic conditions, the retention time for SWM was about 4.75 ± 0.5 mins and for HCTZ it was about 6.15 ± 0.5 mins.

Optimization of Extraction procedure

Extraction procedures tried were Liquid-liquid Extraction (LLE), Protein Precipitation (PPTN) and Solid-Phase Extraction (SPE) to extract SWM and HCTZ from spiked blank rat plasma. In LLE, extraction solvents like ethyl acetate, chloroform, n-hexane, diethyl ether, tertiary butyl methyl ether were used to obtain good recovery of analyte as well as IS. Ethyl acetate showed poor extraction efficiency (EE) of SWM upto 38 % and that of HCTZ upto 68 %. Chloroform showed comparatively better recovery of SWM as 58 % and but poor EE for HCTZ upto 42 %. Rest of the used extraction solvents gave comparatively less EE in range 20-42 % for SWM and HCTZ.

Protein precipitation was performed by using acetonitrile and methanol. The method is not as clean as LLE or SPE, but in some of the cases it gives good recovery of analyte. In this set of experiment, the method was slightly modified. 1 mL of organic solvent acetonitrile in one set and in second set methanol was used. After complete protein precipitation i.e. vortex mixing for 1 minute, 800 µL organic layer was evaporated in centrivap at 65° C and the residue was reconstituted with 125 µL mobile phase. The extraction method gave poor peak resolution and interfering peaks with analyte as well IS.

SPE was performed using C18 and DVB-LP cartridges. Samples extracted using C18 cartridge showed less recovery of HCTZ, upto 57%. Also, peaks obtained for SWM were poorly resolved. Whereas, DVB-LP cartridge showed good recovery of analyte and IS both more than 87% and 89% respectively. But there were many peaks interfering with analyte and IS. This might be because of unwanted moieties from plasma. Hence, SPE was combined with PPTN and to reduce the interference from plasma, plasma was acidified with conc. OPA. The washing step when performed with 0.1 % formic acid instead of distilled water there was no colored residue observed in evaporated methanol after elution. This step excluded microcentrifugation of samples after reconstitution. This gave lesser interference as well as good resolved sharp peaks for SWM and HCTZ in the selected ratio of mobile phase.

Selection of Internal standard

For quantitative bioanalysis using RP-HPLC, it is necessary to employ an appropriate IS that would mimic the analyte in the entire sample extraction, chromatographic elution and UV-visible detection. In modern hyphenated techniques like HPLC-MS/MS, a stable isotope labelled IS that meets above criteria should be used ideally. Taking cost, difficulty in synthesizing stable isotope labeled compounds and difficulty in separation of analyte and

isotope labelled analyte as IS using method like RP-HPLC-UV-Visible in consideration, several other compounds were screened to find suitable IS. The reason why isotope labelled analyte was not used as IS in current method is beside mass, it will mimic the analyte completely in chemical and physical properties and the detection is on the basis of absorbance in UV-Visible detector at 237 nm. UV-Visible detector does not give idea about the mass of the analyte getting detected.

System Suitability

The system suitability was carried out by injecting (n = 6) freshly prepared 1 µg/mL combination solution of SWM and HCTZ. The system suitability was evaluated by calculating % R.S.D. for areas and retention times obtained for SWM and HCTZ in experimental setup. On calculation % R.S.D. was found to be 3.19 and 0.30 for area ratio values and retention times respectively (**Table 01.**).

Table 01. System Suitability

Sample code	Injection Number	Area Ratio	Retention Time
SYS01	1	4.03	4.787
	2	3.88	4.812
	3	3.89	4.809
	4	3.88	4.779
	5	3.67	4.813
	6	3.78	4.795
Mean		3.8550	4.7992
S.D.		0.1231	0.0143
% R.S.D.		3.19	0.30
criteria		% R.S.D. ≤ 5	% R.S.D. ≤ 2

Selectivity and Sensitivity

Fig. 03. represents the chromatograms obtained from analysis of blank plasma, *in vivo* sample at 1.50 hr. after oral administration of LESD and MQC level with IS. Absence of significant peaks at the retention time of the analyte and IS indicated high selectivity of developed method.

Current method was found to be sensitive with LOD and LOQ of 25 ng/mL and 50 ng/mL respectively. This indicates that proposed method is sensitive enough to determine lower concentrations of SWM from samples effectively.

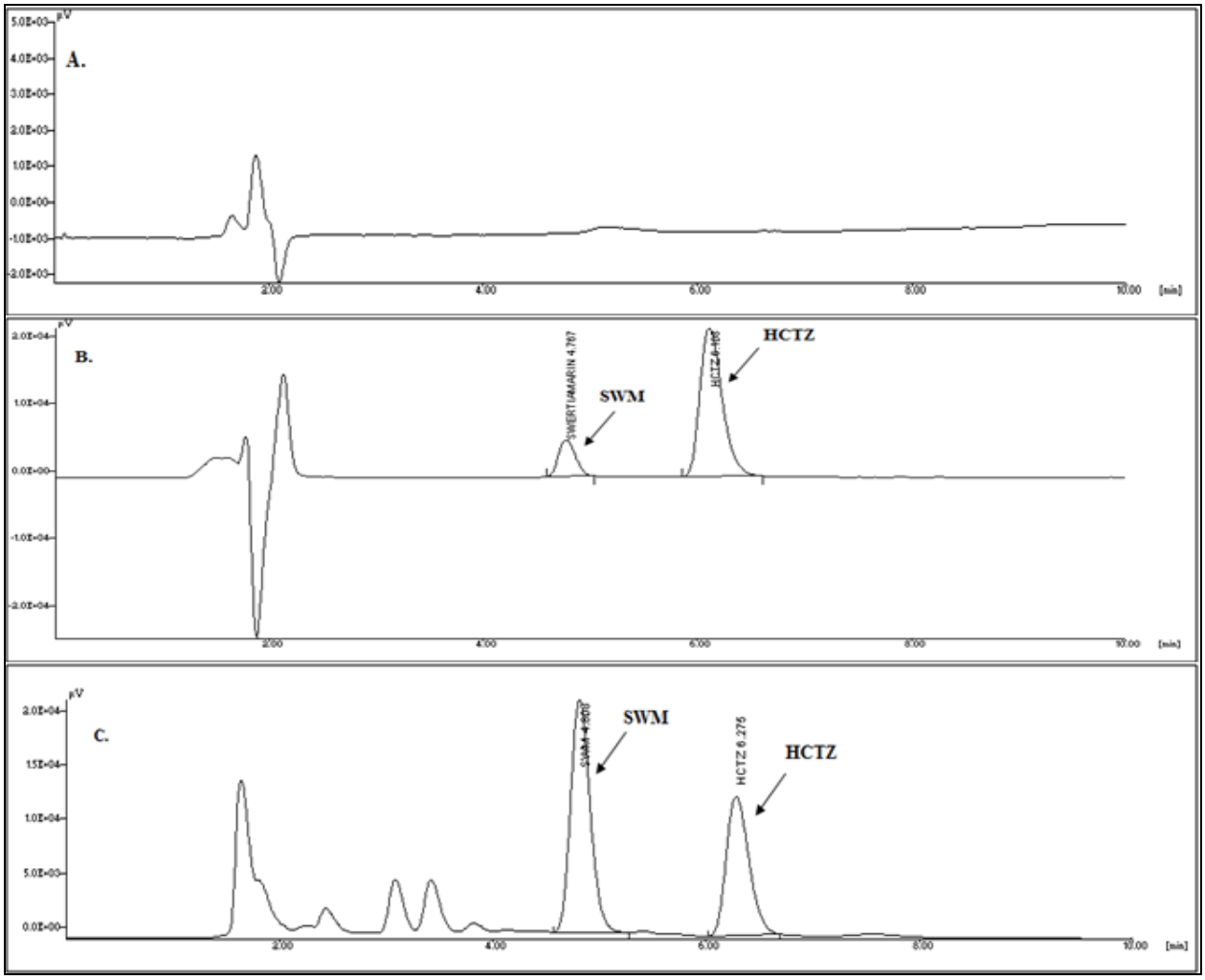

Fig. 03. Representative RP-HPLC chromatograms of SWM and HCTZ obtained from analyzing A. blank rat plasma, B. plasma sample obtained at 1.50 hr after oral administration of LESD and C rat plasma spiked with 7500.00 ng/mL SWM.

Linearity (Calibration curve)

The typical regression equation for eight-point calibration curve (50.0 to 40,000.0 ng/mL) obtained by least squares regression for SWM was y = (0.0005 ± 0.0001) x + (-0.0032 ± 0.0018), r^2 = (0.9988 ± 0.0006), where y represents ratio of analyte peak area to that of IS and x represents the plasma analyte concentration. Further, the results presented in **Table 02.** signify that the calibration curve has a good linear detector response over SWM concentration range.

Table 02. Summary of mean back-calculated concentrations for calibration standards of SWM present in rat plasma (n=4)

Nominal concentration (ng/mL)	50.00	100.00	500.00	1000.00	5000.00	10000.00	20000.00	40000.00
Mean calculated concentration (ng/mL)	49.07	99.87	497.63	958.04	4456.88	9789.45	21195.61	43842.66
± S.D.	3.00	7.69	34.12	91.69	131.53	318.54	576.91	686.76
% R.S.D.	6.11	7.70	6.86	9.57	2.95	3.25	2.72	1.57
% R.E.	-1.87	-0.13	-0.47	-4.20	-10.86	-2.11	5.98	9.61

Precision and Accuracy

Table 03. represents a summary of accuracy and precision results, which are within the acceptable range, indicative of the method being accurate and precise. The data obtained indicates current method has a satisfactory precision, accuracy and reproducibility.

Table 03. Summary of inter-day precision and accuracy for quality control samples of SWM spiked in rat plasma

Nominal concentration (ng/mL)	400.00	7500.00	30000.00
Inter-day assay (n = 6 replicates at each concentration, 3 days)			
Mean ± S.D. (ng/mL)	362.02 ± 16.97	6808.60 ± 268.89	28852.71 ± 2058.27
Precision (% R.S.D.)	4.69	3.95	7.13
Accuracy (% R.E.)	-9.49	-9.22	-3.82
Intra-day assay (n = 6 replicates at each concentration)			
Mean ± S.D. (ng/mL)	384.32 ± 33.41	7187.43 ± 478.98	28921.90 ± 2504.77
Precision (% R.S.D.)	8.69	6.66	8.66
Accuracy (% R.E.)	-3.92	-4.17	-3.59

Dilution Integrity

The % RSD and % Nominal obtained from 2-fold and 4-fold dilutions of spiked plasma were 2.79, 93.56 and 8.35, 94.66 respectively. These results indicate the acceptability of both 2-fold and 4-fold dilutions prior to analysis of *in vivo* samples.

Recovery and Process efficiency

The overall recoveries and process efficiency of SWM at quality control concentrations were consistent and reproducible, as prescribed in **Table 04.**

Table 04. Overall extraction recovery and process efficiency determination for SWM in rat plasma (n=6)

Compound	Nominal Concentration (ng/mL)	Recovery (REC)		Process Efficiency (PE)	
		Mean % REC (± S.D.)	% R.S.D.	Mean % PE (± S.D.)	% R.S.D.
SWM	400.00	91.23 ± 1.88	2.06	84.48 ± 1.33	1.57
	7500.00	90.36 ± 2.69	2.98	83.33 ± 0.10	0.12
	30,000.00	87.52 ± 1.50	1.71	83.36 ± 0.40	0.48
HCTZ	4000.00	89.60 ± 4.09	4.56	84.07 ± 5.09	6.05

Stability

Table 05. briefly summarizes the results of stability experiments performed for SWM in plasma. The data demonstrate that SWM in blank rat plasma was stable for 4 hrs at room temperature and 24 hrs in the autosampler. Further, SWM in blank rat plasma remained unaffected when the samples were subjected to five freeze-thaw cycles stored at -70 ± 5°C for up to 30 days. Data obtained by dry extract and wet extract stability proved the stability of SWM in both the cases.

Table 05. Summary of stability for quality control samples spiked in blank rat plasma (n=3)

Stability	Nominal concentration (ng/mL)	Mean calculated concentration (ng/mL)	Precision (% RSD)	Accuracy (% Nominal)	% RE
Process/ Autosampler[a]	400.00	405.41 ± 16.10	3.97	101.35	1.33
	30,000.00	31442.57 ± 1518.08	4.83	104.81	4.59
Bench-top[b]	400.00	379.80 ± 52.09	13.72	94.95	-5.31
	30,000.00	32622.60 ± 921.77	2.83	108.74	8.03
Freeze-thaw[c]	400.00	389.83 ± 25.28	6.48	97.46	-2.61
	30,000.00	32148.80 ± 1220.38	3.80	107.16	7.16
Long term[d]	400.00	395.87 ± 33.97	8.58	98.97	-1.04
	30,000.00	29089.70 ± 3487.24	11.99	96.97	-3.13

[a] After 24 h in autosampler at 8° C
[b] After 4 h at room temperature
[c] After five freeze/thaw cycles at -70° C
[d] At -70° C for 30 days

Application to pharmacokinetic study

The developed and validated RP-HPLC-UV-Visible method was used to quantify SWM concentration for *in vivo* samples obtained at different time points after oral administration of

LESD (equivalent to 3 mg SWM) to female wistar rats. SWM was detectable upto 12.0 hrs from rat plasma post-dosing. **Fig 04.**, depicts the plot of mean (± S.D.) plasma drug-concentration-time curve profile for SWM. The major pharmacokinetic parameters obtained were as follows: T_{max}: 1.42 ± 0.26 h; C_{max}: 968.81 ± 132.56 ng/mL, $AUC_{0\text{-}12h}$: 3643 ± 1484.84 ng.h/mL, $AUC_{0\text{-}\infty}$: 3883.28 ± 1484.01, $t_{1/2}$: 4.69 ± 1.40 h, K_{el} : 0.31 ± 0.06 L/h.

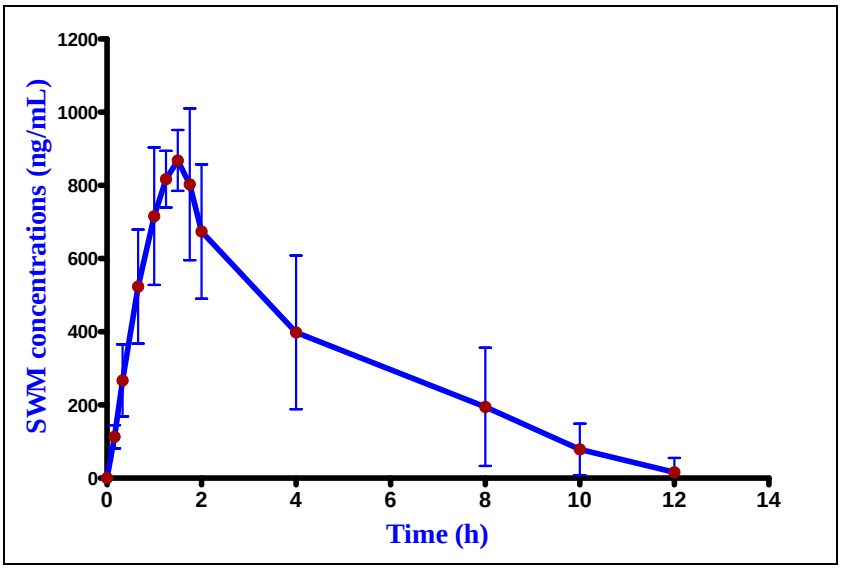

Fig. 04. Mean Plasma drug concentration-time curve profile (Mean ± S.D.) of Swertiamarin (SWM) of after oral administration of LESD (equivalent to 3 mg of SWM) in female wistar rats

CONCLUSION

To the author's knowledge, this is first RP-HPLC-UV-Visible method reported for the quantification of SWM using SPE as sample pretreatment process from rat plasma validated as per US FDA guidelines. This method afforded satisfactory results in terms of sensitivity, selectivity, precision, accuracy, reproducibility and recovery. A low plasma volume (250 mL) SPE sample preparation step resulted in a detection limit of 25.0 ng/mL and quantification limit of 50.0 ng/mL of SWM for the developed method. Further, successful implementation of the method to a pharmacokinetic study in rats demonstrates the feasibility of the method for application to therapeutic monitoring of SWM and *in vivo* studies in human volunteers.

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