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RP-HPLC Method for Simultaneous Estimation of Rutin and Berberine in Bulk and Anti-Urolithiatic Formulation

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Standardization of active phytochemicals is essential for the quality assessment of herbal formulations. In current study a new, simple, rapid and economical RP-HPLC technique was developed and validated for simultaneous estimation of marker compounds rutin and berberine. The mobile phase used was a mixture of acetonitrile, 0.1% orthophosphoric acid in the ratio of 50:50 v/v on a Shiseido Capcell pak C18 column (250 × 4.6 mm, 5 µ) with wavelength detection using UV detector at 265 nm. The time taken for elution of rutin and berberine were 2.27 and 3.31 min, respectively. The linearity range for both rutin and berberine was observed from 5 to 25 µg/mL with correlation co-efficient of 0.997 and 0.999, respectively. The developed method was precise with % relative standard deviation value < 2 for both intra- and inter-day precision. The accuracy of the developed method was accomplished at three concentration levels and the recovered percentage was 99-100.2%. The developed and validated technique was successfully used as standardization tool for the simultaneous determination of markers rutin and berberine in the marketed anti-urolithiatic homeopathic formulation.

Keywords: RP-HPLC, Rutin, Berberine, Anti-urolithiatic formulation.

INTRODUCTION

Urolithiasis is the common painful urological disorder affects around 12% of the world's population at any stage in their life period [1]. It is the condition in which stone formed anywhere in the urinary tract. About 80% of people in the world use herbal drugs for their health care needs [2]. Herbal medicines have been preferred since long times for the treatment of kidney stones compared to conventional medicines due to its low cost, easy availability, lesser chances of side effects and prevention of recurrence of stones [3]. Herbal formulations generally consists of wide range of phytoconstituents but have limited marker compounds. Standardization of marker compounds is essential in order to determine identity, quality, safety and efficacy of the herbal formulations [4]. Various analytical methods have been in use for quality control assessment of herbal medicines.

Anti-urolithiatic formulation (clear stone drops) is a poly herbal formulation, widely used in the treatment of kidney stone disease in homeopathy system of medicine. The formulation composed of herbs like *Berberis vulgaris*, *Sarsaparilla* (*Smilax officinalis*), *Ocimum canum*, *Solidago virgaurea*, *Pareira brava* (*Chondrodendron tomentosum*) and *Senecio aureus* (*Packera aurea*). *Berberis vulgaris* is one of the widely used herbs as a medicine in various traditional systems. The main bioactive compound of herb is berberine (BBR, Fig. 1a) [5]. Traditionally berberine is reported as anti-inflammatory, antioxidant, antimicrobial, antispasmodic, *etc.* [6]. Rutin (RTN, Fig. 1b) is one of the bioactive flavonoid present in most of the herbs of the selected formulation and is reported as antioxidant, antibacterial, anti-inflammatory, antispasmodic, *etc.* [7,8].

Literature review shows that several analytical techniques were reported for estimation of rutin and berberine independently and combination with other phytoconstituents in various herbs and formulations [9-16]. However, there is only single reported HPLC method for the simultaneous estimation of rutin and berberine [17] but the method had longer retention time and poor sensitivity. Therefore, the present study was focused on development of more reliable, sensitive, accurate method over reported technique. The developed technique was also planned to extend for the simultaneous estimation of rutin and berberine in a marketed anti-urolithiatic homeopathic formulation (clear stone drops).

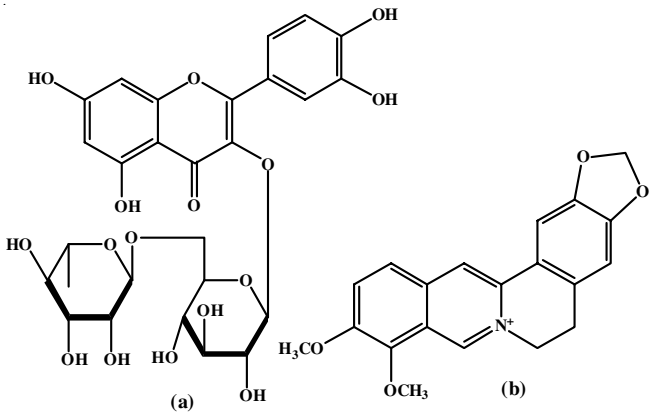

Fig. 1. Structure of (a) rutin and (b) berberine

EXPERIMENTAL

Rutin and berberine standards were purchased from Yucca Phytochemicals Pvt. Ltd, Mumbai, India. HPLC water was purchased from Thermo-Fisher Scientific India Pvt. Ltd., India. Commercial homeopathic formulation (clear stone drops) was bought from a local market. HPLC grade methanol, acetonitrile, potassium dihydrogen phosphate, orthophosphoric acid, acetic acid, potassium hydroxide of AR grade were purchased from Merck specialties Pvt. Ltd., India.

Equipments: RP-HPLC technique was developed using Shimadzu HPLC system with a binary gradient pump (LC-20AD) and UV detector (SPD-20A). The sample was injected using a 20 µL fixed loop Rheodyne injector. LAB-INDIA UV/visible spectrophotometer 3092 used for detection of wavelength.

Determination of absorbance maxima: Each 10 µg of rutin in methanol and berberine in acetonitrile standard solutions were prepared individually and scanned over 200-400 nm, using LAB India UV-visible spectrophotometer. The formed spectra were overlapped (Fig. 2). The wavelength 265 nm was selected as detection wavelength for the determination of rutin and berberine.

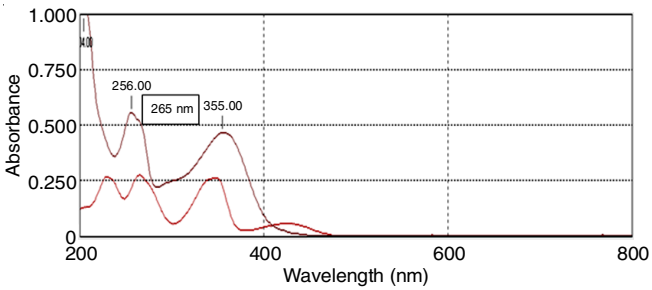

Fig. 2. Overlap UV spectrum of rutin and berberine

Preparation of solutions

Preparation of stock solutions: Accurately weighed 100 mg of rutin and berberine individually into separate 100 mL volumetric flasks, to this add 20 mL of methanol to rutin and 20 mL of acetonitrile to berberine, sonicate for 10-15 min after that the final volume was adjusted using methanol for rutin and acetonitrile for berberine to get a conc. of 1000 µg/mL. From this solution, 100 µg mL^{-1} of rutin and berberine were prepared separately by diluting 10 mL up to 100 mL with mobile phase.

Preparation of working standard solutions: The working standard solution of combination of rutin and berberine (10 µg/mL) was prepared by further diluting 10 mL of the stock solutions of rutin and berberine 100 µg mL^{-1} with the mobile phase up to 100 mL.

Preparation of the mobile phase: Acetonitrile and 0.1% orthophosphoric acid were combined in a 50:50 %v/v to get the mobile phase. Before use, the mobile phase was filtered through 0.45 µm Millipore membrane filter and sonicated. Before injecting solutions, column was stabilized with the mobile phase for 30 min.

Preparation of the test sample solution: About 5 mL of clear stone drops marketed sample was transferred into a separating funnel containing 15 mL of mobile phase. The solution mixture was shaken for 10 min and kept aside for phase separation. The organic phase was separated and was sonicated for 15 min and filtered through 0.22 µ nylon filter. The filtrate was injected in to the system by using the developed method and the chromatogram was recorded at 265 nm.

Method validation: According to ICH recommendations, the developed technique was validated using various parameters such as system suitability, specificity, linearity, accuracy, precision, limit of detection, limit of quantification, ruggedness and robustness.

System suitability: The system compatibility of the developed HPLC technique was confirmed by performing six replica injections of working solution of 10 µg/mL. Each of six replicas injected in to the system and analyzing each for calculation of theoretical plates number (NTP) and tailing factor (T) and resolution (R_s) from the chromatographic data.

Specificity: The chromatograms were recorded for standard, sample and blank solutions under optimized conditions. The method's specificity was determined by evaluating standard chromatograms with blank and sample chromatograms for additional peaks.

Linearity: Calibration curves were used to determine the correlation between concentration (on *x*-axis) and peak area (on *y*-axis). Linearity is expressed by correlation coefficient, should be > 0.99. For linearity determination, prepare both rutin and berberine standard solutions in conc. range of 5-25 µg mL^{-1} and injected into system. The linearity plot was established by 5 replicate injections at all concentration levels.

Accuracy: The proposed method's accuracy was estimated by performing recovery studies in three replicates by spiking at 50, 100, 150% levels.

Precision: Precision study was conducted by intra-day and inter-day assay, expressed in terms of %RSD. It was carried out by determining the sample responses for six replicate injections of one concentration level (15 µg/mL) prepared accordingly described in preparation of solutions. Injected into the system, record the responses for both inter-day and intra-day study.

Limit of detection and limit of quantification: Limit of detection and quantification were used to evaluate the sensitivity of the proposed method. These were determined based on signal to noise ratio technique.

Robustness: The developed method was analyzed by introducing slight and intentional changes in the chromatographic conditions. Variations introduced in composition of mobile phase and rate of flow to check the robustness of the method. The rate of flow was changed from 1 mL min^{-1} to 0.9 mL min^{-1} and 1.1 mL min^{-1}. The variation in organic phase mobile phase composition varies from 50:50 ratios to 52:50 and 48:50.

RESULTS AND DISCUSSION

In literature, there is only single RP-HPLC technique was published earlier for simultaneous determination of rutin and berberine, but the reported method had limitations of long retention time and poor sensitivity. The aim of the present study is to develop a new, simple, sensitive, rapid and economical method for simultaneous determination of rutin and berberine in bulk phytochemicals and the developed technique is applied to poly herbal formulations. Different trails were conducted to optimize the chromatographic conditions. Trails were performed with various mobile phases containing water, methanol, acetonitrile, water, potassium dihydrogen phosphate buffer, orthophosphoric acid and with different columns. After conducting various trials the method shows ideal peak resolution with optimum symmetry for simultaneous determination of rutin and berberine using acetonitrile:orthophosphoric acid 50:50 v/v. The time taken for elution of rutin and berberine were 2.27 min and 3.31 min, respectively. Rutin being more polar in nature makes the selected mobile phase favoured the elution of rutin first when compared to berberine. Optimized analytical conditions are displayed in Table-1.

TABLE-1
OPTIMIZED ANALYTICAL CONDITIONS

Parameters	Optimized conditions
Column	Shiseido Capcell pak C18 column (250 × 4.6 mm, 5 µm)
Mobile phase	Acetonitrile:0.1% orthophosphoric acid (50:50)
Rate of Flow	1.0 mL/min
Injection volume	20 µL
Run time	8 min
Detection wave length	265 nm
Temperature	25 ± 2 °C

As per ICH criteria, a novel, simple, sensitive, rapid and economical RP-HPLC technique was designed and validated. From the specificity study (Fig. 3a-c), it is concluded that there was no interference in the resolution of markers rutin and berberine in the chromatograms, indicating that the developed method is suitable for simultaneous analysis of markers.

During system suitability, studied several factors such as %RSD, NTP, tailing factor (T) and resolution (R_s). The results are displayed in Table-2. The values of proposed method were %RSD of peak area for rutin and berberine were 0.83, 1.04 and theoretical plates 2941, 4412 and tailing factor of 1.07, 1.02 and resolution 5.009. From System suitability results, it is concluded that the optimized method parameters were good and suitable for estimation.

TABLE-2
SYSTEM SUITABILITY RESULTS

Injection	Peak area	
	Rutin	Berberine
Injection 1	373389	743643
Injection 2	376115	733036
Injection 3	376460	725050
Injection 4	370251	727010
Injection 5	370094	738111
Injection 6	376904	725140
Mean	373868.83	731998.33
S.D	3115.76	7671.23
%RSD	0.83	1.04
NTP	2941	4412
Tailing factor	1.07	1.02
Resolution	–	5.009

From linearity data (Table-3a,b), it was observed that a linear relationship was established in the conc. range of 5-25 µg mL^{-1} with correlation coefficient value of 0.997 and 0.999 for rutin and berberine, respectively, indicating that standard samples concentration and peak area were well correlate and the proposed HPLC technique is linear. The concentrations of marker compounds in the test sample were calculated by substituting sample peak area in linearity equation shown in Table-3b.

TABLE-3a
LINEARITY DATA OF RUTIN AND BERBERINE

Conc. (µg/mL)	Rutin		Berberine	
	Peak area* ± SD	%RSD	Peak area* ± SD	%RSD
5	163389±158.27	0.09	343643±185.23	0.05
10	323173±144.28	0.04	720450±312.26	0.04
15	505419±509.97	0.10	1137318±855.36	0.07
20	658358±245.68	0.03	1488378±547.36	0.03
25	796242±570.42	0.07	1887752±325.22	0.01

*Mean of 5 determinations

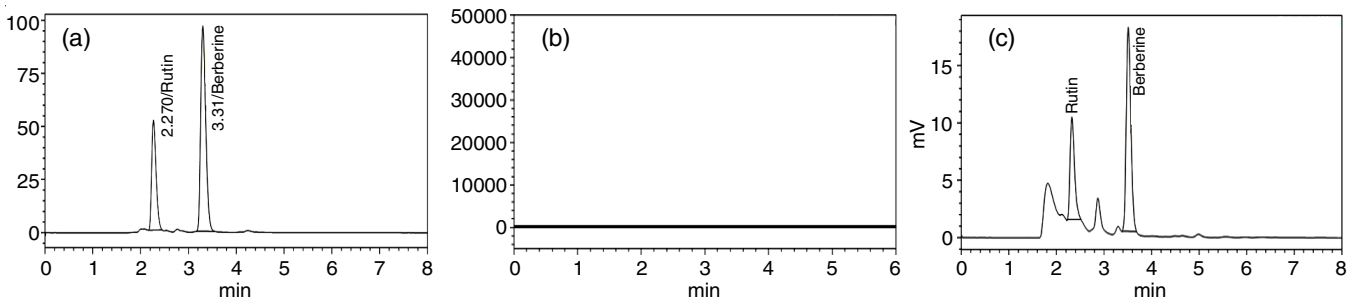

Fig. 3. Chromatogram of standard (rutin and berberine) (a); blank (b) and rutin and berberine in polyherbal formulation (c)

TABLE-3b
LINEARITY OF RUTIN AND BERBERINE

Compound	Conc. (µg/mL)	Correlation co efficient value	Slope	Linearity equation	Concentration in clear stone drops (mg/mL)
Rutin	5-25	0.997	32018	y = 32018x + 9048	1.530
Berberine	5-25	0.999	77123	y = 77123x – 41336	1.009

Absence of additional peaks in the chromatogram indicating that the developed RP HPLC technique is suitable for simultaneous quantification of these marker compounds without any interference of other phytochemicals present in the formulation.

The sensitivity parameters were found out to know about minimal concentration required for quantification of markers in the formulation. LOD and LOQ results of rutin and berberine are given in Table-4. The low levels of LOD and LOQ values of rutin and berberine, confirming that the developed technique was sensitive.

TABLE-4
LIMIT OF DETECTION (LOD) AND
LIMIT OF QUANTIFICATION (LOQ)

Parameter	Rutin (µg mL^{-1})	Berberine (µg mL^{-1})
Limit of detection	0.005	0.001
Limit of quantification	0.05	0.01

The accuracy for the optimized technique was determined by performing recovery studies of spiking at 50, 100, 150% levels. Results are shown in Table-5. In present study, the percentage recovery of rutin and berberine were found to be 99.2 to 100.16% and 99.52 to 100.06% indicating that the developed technique was able to recover markers completely from formulation, no interference with other constituents and makes the method accurate.

Intra-day precision and inter-day precision studies were conducted for 3 replicate injections of 15 µg/mL conc. and results are displayed in Table-6. The proposed method was precise, as evidenced by % relative standard deviation of both intra-day and inter-day precision studies were < 2.

Robustness was tested by variations in rate of flow and mobile phase composition, had no marked effect on the method results and statistical data of results were shown in Table-7, revealed that the developed method was robust.

TABLE-7
ROBUSTNESS RESULTS OF RUTIN AND BERBERINE

Robustness parameters	Variations	%RSD	
		Rutin	Berberine
Flow rate	± 0.1 mL min^{-1}	1.06-1.57	0.99-1.09
Organic phase composition	± 2%	0.65-1.06	0.46-0.87

Conclusion

Analytical techniques have been developed to ensure the quality, safety and efficacy of formulations. It is vital to check the quality of polyherbal formulations due to their complex phytochemical profile. The developed RP-HPLC technique is appropriate for simultaneous determination of berberine and rutin in standard phytochemicals and also in polyherbal formulations. The developed technique was superior in terms of less retention time, low limit of detection, simple sample preparation, good resolution and no interference of other phytoconstituents in the simultaneous estimation of rutin and berberine.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

TABLE-5
ACCURACY STUDIES

Level (%)	Standard added (µg/mL)	Sample added (µg/mL)	%Recovery* ± S.D		%RSD
			Rutin	Berberine	
50	5	5	99.2 ± 0.09	99.52 ± 0.09	0.09, 0.09
100	15	5	100.16 ± 0.45	100.06 ± 0.30	0.29, 0.44
150	25	5	99.65 ± 0.47	99.83 ± 0.39	0.39, 0.48

*Mean of 3 determinations

TABLE-6
INTRA-DAY AND INTER-DAY PRECISION

Concentration (15 µg/mL)	Rutin (peak area)		Berberine (peak area)	
	Intra-day	Inter-day	Intra-day	Inter-day
Injection 1	490338	498360	1120987	1134441
Injection 2	506727	507526	1144667	1144050
Injection 3	504384	499660	1138699	1143144
Injection 4	505859	505239	1116833	1127388
Injection 5	505419	505859	1137318	1116833
Injection 6	505338	490094	1120987	1138111
Mean ± S.D	503010.8 ± 6254.96	501123 ± 6508.19	1134784.333 ± 12315.80	1133994.5 ± 10393.59
%RSD	1.24	1.29	1.08	0.91

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