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Simultaneous Method Development and Validation of Berberine, Rubiadin and 3-O-acetyl-11-keto-β-boswellic acid By Reverse Phase High Performance Liquid Chromatography In Polyherbal Formulation

Research Article

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Abstract

Berberine, Rubiadin and 3-O-acetyl-11-keto-β-boswellic acid (AKBA) are reported mainly for their anti-inflammatory activity among other uses like antidiarrheal, anti-fungal, antitumor, anti-oxidant, anti-diabetic and immunomodulation. The polyherbal gel formulation contains hydrolalcoholic extracts of *Berberis aristata* DC., *Rubia cordifolia* L., *Boswellia serrata* Roxb. And it is claimed for anti-inflammatory and anti-bacterial activity. Hence, the current work was planned to estimate Berberine, Rubiadin and AKBA in the polyherbal formulation. A Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method was planned for the simultaneous estimation of Berberine, Rubiadin and AKBA in accordance with International Council for Harmonisation (ICH) guidelines. The chromatographic separations of the active ingredients were achieved in the C18 column (250×4.6 mm;5 µm), the mobile phase consisted of acetonitrile: phosphate buffer pH 3.5 (20:80 %v/v), the flow rate was adjusted to 1 ml/min and Ultra Violet (UV) detection was carried out at 261 nm. The retention time for Berberine, Rubiadin and AKBA was 6.76, 8.37 and 5.76 min, respectively. The detector showed linear responses for all three compounds in the concentration range 1-5 µg/ml, which had a good correlation coefficient (0.996). The developed method was also validated as per ICH guidelines. This RP-HPLC method is simple, precise, sensitive and accurate. It can be used for quality control of raw materials and evaluation of herbal formulations containing Berberine, Rubiadin and AKBA.

Key Words: *Berberis aristata DC., Rubia cordifolia L., Boswellia serrata Roxb.,* Anti-inflammatory, Anti-fungal.

Introduction

Berberine is an alkaloid which have been used in *Ayurveda* and Chinese medicines from ancient time as *Berberis aristata* DC (*Daruhaldi*). It is also present in many plants like *Hydrastis canadensis* L. (goldenseal), *Coptis chinensis* Franch., and *Berberis aquifolium* Pursh. (Oregon grape). Berberine extracts have been reported for antimicrobial activity against a range of microorganisms viz., bacteria, viruses, fungi, protozoans, helminths, and chlamydia. In China, berberine is used for the treatment of diarrhea.

Root of *Berberis aristata* DC. contains alkaloids that are berberine, arachine dihyrokarachine, berbamine, epiberberine, palmatine, oxycanthine, dehydrocaroline, oxyberberine, aromoline, jatrorhizine and columbamine. In most of *Berberis* species, berberine and berbamine are the most biologically active markers (1). Berberine is pharmacologically active as anti-diabetic, bactericide, anti-inflammatory, antidiarrheal and anti-fungal properties (2).

Rubiadine is dihydroxy anthraquinone which has antioxidant properties and it is isolated from R*ubia cordifolia* L. alcohol extract (3). Pharmacological activity of *Rubia cordifolia* L. stem and root has been reported as blood purifier, antitumor, antioxidant (4).

Seven anthraquinones were isolated from the ethyl alcohol extracts of the roots of *Rubia cordifolia* L. Six of them were identified as 2- methyl-1,3,6-trihydroxy-9,10-anthraquinone, 1-hydroxy-9,10-anthraquinone, 1,2,4-trihydroxy-9,10-anthrequinone, 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone-3-O-beta-D-glucoside, 1,2-dijhydroxy-9,10-anthraquinone-2-Obeta-D-xylosyl(1-6)-beta-D -glucoside and 1,3-dihydroxy-2-hydroxymethyl1- 9,10-anthraquinone-3-O-beta-D- xylosyl(1, 6)-beta-D-glucoside. The structure of seventh compound was elucidated as 2-methyl-1,3,6-trihydroxy-9,10-anthraquinone-3-O-beta-D-xylosyl(1-2)-beta-D- (6'-O-acetyl) glucoside (5).

Rubia cordifolia DC. (*Manjishtha*) belongs to Rubiaceae family and it is also known as a *Madder* or Indian *Madder*. It is considered as a healthy herb in ayurvedic medicinal system (6). Rubiadin (0.5 mg/kg)

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was effective in granuloma induced by cotton pellet concerning the granuloma and transudate formation amount. Rubiadin's anti-inflammatory effects was associated with a significant IL-1β decrease in this model. The results suggest that Rubiadin is a natural compound can possess significant peripheral anti-inflammatory impacts (7).

AKBA (3-O-acetyl-11-keto-β-boswellic acid) is a natural gum resin extracted from *Boswellia serrata* Roxb., it was recently shown to possess positive therapeutic effects in inflammatory gut diseases. *Boswellia serrata* Roxb. extract containing AKBA considerably attenuated tissue injury scores. Oral medical care with the *Boswellia serrata* Roxb extract (containing AKBA) significantly reduces megascopic and microcirculatory inflammatory options ordinarily related to NSAID administration, indicating that the anti-inflammatory actions of the *Boswellia* extract in IBD (Inflammatory Bowel Diseases) could also be partially due to boswellic acids (AKBA) (8).

Boswellic acids are mixture of triterpenic acids and it is found from the natural resin of incense tree and known for its effectiveness for the treatment of chronic disease together with peritumor edema. Boswellic acids are extensively studied for variety of activities including antitumor, immunomodulatory and inflammatory diseases (9).

Berberine, Rubiadin and AKBA were selected as biological markers for *Berberis aristata* DC, *Rubia cordifolia* L.and *Boswellia serrate* Roxb. respectively (10-16). There are reported methods available for determination of Berberine, Rubiadin and AKBA independently. There is no Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method reported for the simultaneous estimation of Berberine, Rubiadin and AKBA in any polyherbal formulations. Hence, the current work was planned to estimate Berberine, Rubiadin and AKBA in the polyherbal gel formulation. An RP-HPLC was planned to develop for validation and simultaneous estimation of Berberine, Rubiadin and AKBA in accordance with International Council for Harmonisation (ICH) guidelines.

Materials and Methods

Reference standards

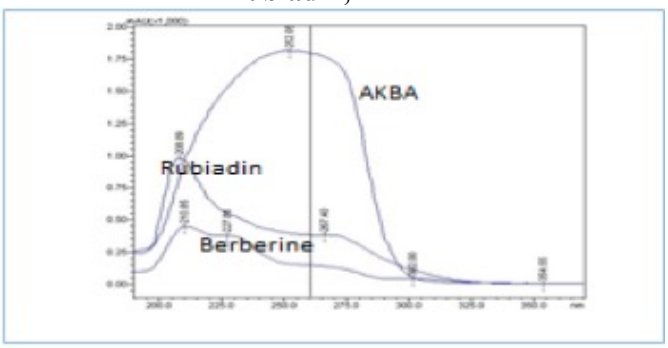
Berberine, Rubiadin and AKBA were procured from Natural Remedies, Bengaluru, Karnataka, India.

Chemicals

- Acetonitrile, distilled water and methanol used were of HPLC grade, procured from Merck India
- Potassium dihydrogen phosphate and O- phosphoric acid were of HPLC grade, procured from Merck India
- *Selection of wavelength*

Primary stock solution (20 µg/ml) of each were prepared for all three markers by dissolving in methanol and diluted further to make a secondary stock solution at 3 µg/ml each to obtain a UV-visible spectrum. The standard solutions of Berberine (3 µg/ml), Rubiadin (3 µg/ml), AKBA (3 µg/ml) in methanol were examined individually in the UV between 200 to 400 nm and the overlay spectra were recorded. Wavelength selected for detection was 261 nm.

Figure No.1: Overlay spectra of Berberine, Rubiadin, AKBA



Instrumentation

HPLC (Model: Agilent 1200 spinchrome)

All reference standards were weight accurately and calibrated by weighing balance (Shimadzu).

Chromatographic conditions of developed method.

Table No.1: Optimized chromatographic condition for developed method

Parameters	Specification
Mobile phase	Acetonitrile: : Phosphate Buffer pH 3.5 (20:80 %v/v)
Pump mode	Isocratic
Column	C18 - (250x 4.6) mm; 5µm
Flow rate (ml/min)	1 ml/min
Run Time (min)	15 min
Volume of injection (µl)	20
Detection wavelength (nm)	261

Preparation of Phosphate Buffer Solution

Accurately weight 0.07 gm of anhydrous potassium dihydrogen O-phosphate was transferred to 500 ml graduated flask and approximately 450ml of HPLC water was added into it. The mixture was kept for ultra-sonication for ten minutes to obtain complete dissolution. 0.25 ml of O-phosphoric acid was added to it and final volume was made upto 500 ml with water. The resulting solution was filtered through 0.45 micro meter nylon filter paper by vacuum filtration assembly.

Preparation of Reference standards solution

Accurately weighed 2 mg of Berberine, Rubiadin and AKBA were taken separately in a 100 ml volumetric flask and added with 50 ml of HPLC grade methanol in graduated flask and sonicated solution for 10 minutes and made up to 100 ml by methanol. For working standard concentration range, 20 µg/ml standard stock solution was used.

Preparation of Sample Solution

Hydroalcoholic extracts of *Berberis aristata* DC, *Rubia cordifolia* L.and *Boswellia serrate* Roxb was formulated to a polyherbal gel formulation with carbopol 934 (17). One gram of gel in 100 ml graduated

flask was taken and added with 50ml methanol and sonicating solution for 5 minutes and made up to 100 ml with methanol and shaked. The solution was filtered by 0.45µm filter paper and resultant solution was diluted with methanol for analysis.

Analytical method developemtn

Linearity

Calibration curve was created by plotting peak area v/s concentration. Linearity was evaluated by different concentrations (1, 2, 3, 4, 5 µg/ml) of Berberine, Rubiadin and AKBA and five such samples were evaluated (n = 1 × 5).

Precision

Results for interday and intraday precision were calculated and stated as % relative standard deviation.

Repeatability

Mixture of standard solution at 100% level (2 µg/ml) was evaluated six times, area of peaks were calculated and % RSD was calculated to find out the repeatability of the method.

Intraday and Interday precision

Mixture of reference standard solution at different level (50%, 100%, 150%) of concentration were analyzed for intraday precision at different days for the finding of interday precision and % RSD was calculated.

Robustness

By variable changing the mobile phase mixture (+2 and -2), pH change in mobile phase (+2 and-2) and flow rate change (+2 and-2), the effects on the results were examined. Robustness of the method was calculated at concentration levels 2 µg/ml.

LOD and LOQ

- LOD: The determination of a single analytical process is the minimum amount of standard drugs in a sample which can be determined.
- LOQ: The quantification limit of a single analytical process is the minimum amount of standard drugs in a sample which can be quantifiable determined.
- For the proposed method LOD and LOQ were calculated by following equation:

$$LOD=3.3x\ \sigma/s \qquad LOQ = 10\ x\ \sigma/s$$

where σ = Standard deviation of the response S =Slope of calibration curve

Specificity

The specificity of developed method was investigated with injecting of blank samples of mobile phase to demonstrate the absence of interference in standard samples.

Accuracy

Accuracy of the assay was observed by recovery studies at different concentration levels (50%, 100%, and 150%), i.e., 1, 2, 3 µg/ml, and three replicate samples from each concentration were insert with known concentration of sample solution. The % recovery of added standard mixture and % RSD were calculated for each of the replicate samples.

Results and Discussion

Linearity

The linearity of calibration curve of Berberine, Rubiadin and AKBA was established in the range 1-5 µg/ml. Results are shown in table no.2.

Table No.2 Linearity data of reference compounds

Sr. No.	Reference compounds	R^2 Value	Equation
1	Berberine	0.997	Y=873.44X+ 10.2
2	Rubiadin	0.996	Y=337.36X+25.34
3	AKBA	0.996	Y=848.97X+19.53

Berberine, Rubiadin and AKBA showed linear response from 1-5 µg/ml. Correlation of coefficient of Berberine, Rubiadin and AKBA were found to be 0.997, 0.996, and 0.996 respectively.

System Suitability

The system suitability parameters of optimized method was calculated by different chromatographic parameter like number of theoretical plate, resolution (Rs), tailing factor (T) from chromatogram of reference standard solutions. Results are shown in table no.3.

Table No.3 System suitability parameters of developed method

Parameters	Observation			Specification
	Berberine	Rubiadin	AKBA	
Retention time	6.76	8.37	5.76	--
Resolution (Rs)	3.50	4.52	--	Rs >2
Theoretical plate(N)	7280	7337	8176	≥2000
Tailing factor (T)	1.01	1.05	1.02	<1.5

Above system suitability parameters are within range and as per ICH guideline.

Precision

Repeability precision was performed by injecting same concentration for 6 time in a day and %RSD obtained was between 0.73 to 1.16.

Intraday precision was measured twice in a day and %RSD ranged from 0.24 to 1.75 and interday precision were measured in two continuous days and %RSD obtained between 0.24 to 1.64. Results are shown in table no.4.

Table No.4 Precision study of developed method

Parameters	% RSD		
	Berberine	Rubiadin	AKBA
Repeability (n=6)	0.73	1.16	0.89
Intraday(n=2)	0.55	0.67	0.65
Interday(n=2)	0.50	1.08	0.90

Results show that %RSD of Berberine, Rubiadin, AKBA was less than 2 as per ICH acceptance criteria. So, the developed method for estimation of Berberine, Rubiadin, AKBA is precise in quality.

Robustness

Robustness of optimized method was studied by minor changes in the different experimental conditions like change in composition of mobile phase (+0.2 and-0.2), change in mobile phase pH (+2 and-2) and change in flow rate timing (+0.2 and-0.2), the effects of different experimental conditions were examined. Results are shown in table no.5.

Table No.5: Robustness study of developed method

Parameters	Changes	% RSD		
		Berberine	Rubiadin	AKBA
Mobile phase composition	3.5 ±0.2 ml	1.18 1.14	1.07 1.30	0.91 0.56
pH change in mobile phase	20: 80 ±2 (v/v)	1.07 0.74	1.07 0.74	0.61 0.76
Flow rate change	1±0.2 ml/ min	1.99 1.88	1.06 1.30	1.18 0.66

Results showed that the minor changes in the experimental conditions remained unaffected and %RSD < 2, indicating that the proposed method was robust.

LOD and LOQ

LOD and LOQ were calculated and results are shown in table no.6.

Table No.6 LOD and LOQ study of developed method

Parameters	Berberine	Rubiadin	AKBA
LOD	0.11	0.76	0.22
LOQ	0.03	0.25	0.07

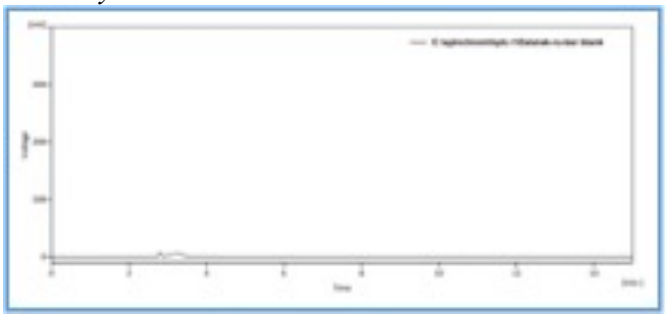
Results shows that the optimized method is very sensitive.

Specificity

With the compression of the chromatograms of standard solution and blank, specificity was determined. It was shown that there were no peaks found at the retention time (RT) of standards (Figure 2).

Figure No.2 Chromatogram of Blank

Accuracy



Accuracy of assay was determined as relative recovery at different concentration levels in the range of 98.87 to 100.25 % and over all %RSD value is < 2. Results are shown in table No.7.

Table No.7 Accuracy study of developed method

Parameters	% Recovery study		
	Berberine	Rubiadin	AKBA
50%	100.05	99.64	99.29
100%	99.26	99.90	98.87
150%	99.25	99.45	99.07
Average	99.50	99.66	99.07

Result shows that % recovery of Berberine, Rubaidin and AKBA was between 98-102% as per ICH acceptance criteria.

Conclusion

The current developed HPLC method can be effectively used to standardise and estimate Berberine, Rubiadin and AKBA (3-O-Acetyl-11-keto-β-Boswellic Acid) containing herbal formulations, simultaneously. The developed HPLC method was validated as per ICH R1 (Q2) guidelines. The method well-tried to be sensitive, precise, accurate and reliable.

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Conflict of interest

No

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