

STANDARDIZATION OF AN AYURVEDIC DRUG - MADHUMEHA KUSUMAKAR RASA BY HPTLC

K S Thakur¹, P P Pimpalkar², M D Gawhankar³, R V Gudi⁴

^{1,4}Shree Dhootapapeshwar Limited,

^{2,3}Shree Dhootapapeshwar Ayurvedic Research Foundation,
Veer Savarkar Chowk, Pangel, Navi Mumbai, Maharashtra, India

ABSTRACT

Madhumeha Kusumakar Rasa (MKR) is an Ayurvedic medicine having ingredients of *Vasantkusumakar Rasa*, *Shuddha Shilajeet*, *Jasad Bhasma*, Extract of *Mamajjaka* (*Encostemma Littorale*), *Haridra* (*Curcuma longa*), *Amalaki* (*Embllica officinalis*) and *Guduchi* (*Tinospora cordifolia*) indicated for complications of *madhumeha* (Diabetes). The purpose of this work was to develop and validate HPTLC method for quantification of marker compound Swertiamarin which is expressed in formulation through one of its major ingredient (*Pradhan dravya*) *Mamajjaka* (*Encostemma Littorale*). The formulation was subjected to methanol extractions and extracted samples were applied on TLC plate precoated with Silica Gel 60GF₂₅₄. The detection and quantification was performed at a wavelength of 240 nm. The method validation was carried out as per ICH guidelines. Calibration curve plotted was found to be linear in the range of 200 – 900 ng. The linear regression equation was found to be $Y = 3.50 X + 670.5$, while correlation coefficient (r^2) was 0.9996 with high reproducibility and accuracy. LOD and LOQ were found to be 67.8 and 205.6 ng respectively. *Madhumeha Kusumakar Rasa* samples MKR-1, MKR-2 and MKR-3 were found to contain Swertiamarin 15.08 mg/tab, 14.95 mg/tab and 14.30 mg/tab respectively. This method was thus found to be linear, precise and accurate for quantitative determination of Swertiamarin in MKR.

Keywords: *Madhumeha Kusumakar Rasa*, *Mamajjaka*, *Swertiamarin*, HPTLC.

INTRODUCTION

Ayurved is one of the widely practiced and recognized systems of medicine in India. This traditional system of medicine recommends usage of herbs and minerals in formulations for holistic health care and cure to various ailments and health disorders.

Madhumeha Kusumakar Rasa has been indicated in various complication of *Madhumeha* like *Madhumehajanya netravikar*, non heal-

ing diabetic wound, polyuria, *naktamootrata* etc. This formulation consist of ingredients like *Suvarnayukta Vasant Kusumakar Rasa*, *Mamajjaka Ghana*, *Amalaki*, *Haridra* which are reported for reducing blood and urine sugar effectively due to their *kapha* and *meda pachak* action^[1].

Encostemma Littorale Blume is taxonomically classified in family Gentianace un-

der order Gentianales. This herb *Enicostemma Littorale* has been referred in Ayurvedic text for various ailments like antacid, antipyretic^[2, 3] and diabetes^[4]; leaf of *Enicostemma Littorale* is known to show hepatoprotective activity^[5,6]; treatment of type II diabetes by reducing blood glucose levels and improving blood pressure, lipid profile and kidney function^[7,8]; Aqueous extracts of *A. marmelos* in combination with *Enicostemma Littorale* were studied in diabetes induced animals and found to reduce hyperglycemia^[9]. Extracts of *mamajjaka* (*Enicostemma Littorale*) showed protective effects in diabetic neuropathy^[10].

Marker compound Swertiamarin is known to be present in *Enicostemma Littorale* Blume^[11], *Mamajjaka* (*Enicostemma Littorale*) Ghana which is one of major constituent of MKR drug.

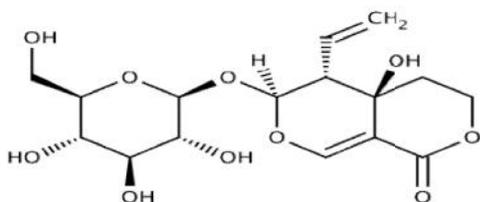


Fig. 1 Structure of Swertiamarin

Due to complex nature of product having multi herbo-mineral ingredients, analytical method developed needs to be systematically optimized and validated for Accuracy, Precision, Linearity, LOD and LOQ as per ICH guidelines^[12].

MATERIALS AND METHODS

The formulation of *Madhumeha kusumakar Rasa* Tablet 3 batches were procured from stockiest of Shree Dhootapapeshwar Ltd. These formulations were coded as MKR-1, MKR-2 and MKR-3. Reference standard (RS) Swertiamarin of purity 95.40 % (CAS No, 17388-39-5) was

used for the study along with other chemicals, solvents and reagents of analytical grade.

Preparation of Mobile Phase

The mixtures of several mobile phases were tried to separate spot of Swertiamarin from other spots and get stable peak. The solvent system Ethyl acetate: Methanol: Water (7.7 : 1.3 : 0.8 v/v/v) was selected for estimation of Swertiamarin, which gave good resolution. Good chromatogram was attained with Rf value 0.35 ± 0.04 . The wavelength of 240 nm was used for quantification of sample.

Preparation of Standard solution

A stock solution of Swertiamarin (0.1 mg /ml) was prepared by dissolving 5 mg of reference standard in 50 ml of methanol.

Instrumentation and Chromatographic conditions

HPTLC Instrument Camag, Linomat 5 fitted with TLC Scanner 4, Wincat Software was used for chromatographic analysis of sample. Twin trough chamber was used for development of HPTLC plate. Photo documentation cabinet fitted with High Resolution camera was used for capturing images at different wavelengths. Densitometer TLC Scanner 4 equipped with D2 lamp was used to obtain spectra for quantitative determination of compound.

Stationary Phase

Precoated silica gel G60-F₂₅₄ Aluminium sheet (E. Merck, Germany) 20 x 10 mm, thickness layer 0.2 mm was used.

Calibration Curve

With 100 µl syringe, 2 µl, 4 µl, 5 µl, 6 µl, 7 µl, 8 µl and 9 µl of standard solution (0.1 mg/ml) under Nitrogen Stream was applied in form of bands of expected concentration (200 – 900 ng/spot) on Silica pre coated TLC plates.

Plates were developed in Solvent System Ethyl acetate: Methanol: Water (7.7 : 1.3 : 0.8 v/v/v) at temp $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and dried in air. Densitometric Scan was performed in Absorbance mode at wavelength 240 nm using camag TLC Scanner 4. Standard Graph was plotted with peak area Vs concentration of Swertiamarin.

Sample Preparation

Powdered samples of MKR (0.5g accurately weighed) were refluxed on water bath with methanol (2x25 ml) for two times. Combined extracts were concentrated to 25 ml under vacuum and used for analysis.

Method Validation

Method development was performed as per standard ICH guidelines^[4] which included linearity, precision, accuracy, LOD and LOQ. Linearity of method was performed by plotting calibration curves in concentration range of 200

– 900 ng. Precision of method was performed by estimating intraday and interday readings and % RSD (Relative standard deviation) was calculated.

Accuracy of analytical methods was expressed as % recovery. This was estimated by adding known concentration of standard solution to pre analyzed sample solution.

Specificity of method was assessed by confirming the spectra and Rf value of sample matching with the swertiamarin standard. Limit of Detection (LOD) and Limit of Quantification (LOQ) were estimated as per formula: $\text{LOD} = 3.3 \times \text{S} / \text{S}$ & $\text{LOQ} = 10 \times \text{S} / \text{S}$

Where S = Standard deviation, S = Slope

RESULTS

For development of successful method, the first important step is to optimise the mobile phase. Mobile phase optimization was carried out by conducting trials with various combinations of solvent system. Thus mobile phase with solvent system Ethyl acetate : Methanol : Water (7.7 : 1.3 : 0.8 v/v/v) was screened out to give clear, sharp and well defined peak at Rf value 0.35 ± 0.04 in standard and sample (Fig 2 & Fig 3).

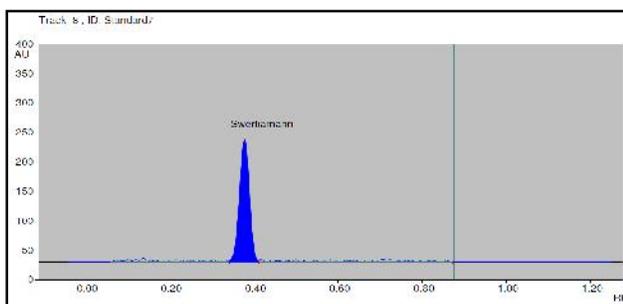


Fig 2: Densitogram obtained from Standard level-7 for Swertiamarin at 240 nm

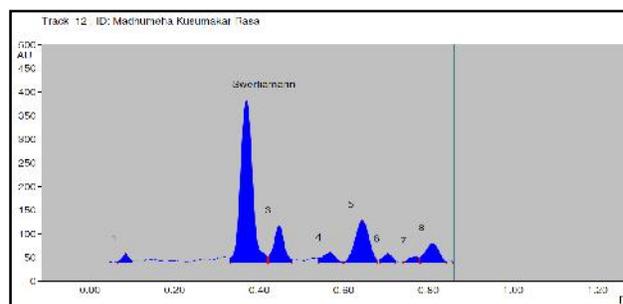


Fig 3: Densitogram obtained from Test solution of Madhumeha Kusumakar Rasa for Swertiamarin at 240 nm

Specificity

It was observed that other constituents present in *Madhumeha Kusumakar Rasa* did not interfere with the peak of Swertiamarin. Thus the proposed method was proved to be specific. The band of standard Swertiamarin observed at Rf

0.35 ± 0.04 matches with *Madhumeha Kusumakar Rasa* samples which is shown in Fig.4. The spectra of standard Swertiamarin corresponded with *Madhumeha Kusumakar Rasa* is shown in Fig.5

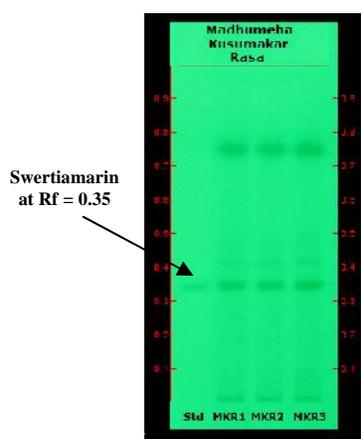


Fig 4: HPTLC plate of Madhumeha Kusumakar Rasa with Swertiamarin standard at 254 nm

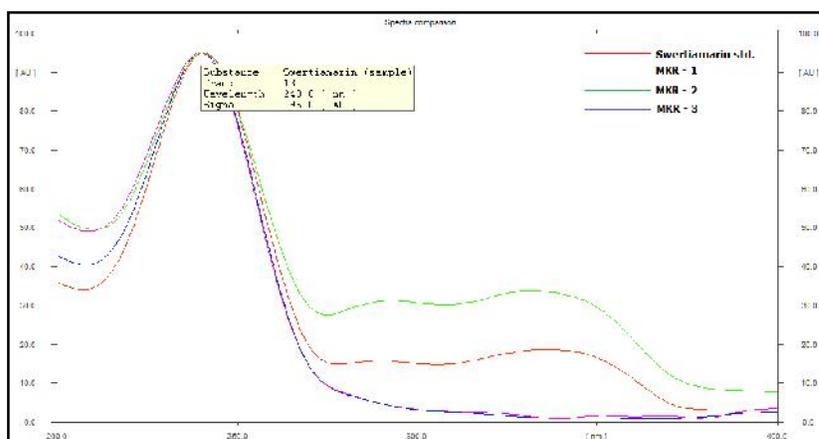


Fig 5: Overlay Spectra of Standard Swertiamarin & Madhumeha Kusumakar Rasa showing wavelength maxima at 240 nm

Linearity

Calibration curve plotted was observed to be linear in the concentration range of 200 – 900 ng. The linear regression equation was found to be $Y = 3.50 X + 670.5$ for standard and correlation coefficient (R^2) observed to be 0.9996 (Fig.6 & Table 1).

#	Parameters	Observed Values
1	Linearity range (ng/spot)	200 - 900
2	Correlation coefficient	0.9996
3	Linear Regression equation	$Y = 3.50 X + 670.54$
4	Slope \pm SD	3.5
5	Intercept \pm SD	676.5
6	LOD (ng/spot)	67.86
7	LOQ (ng/spot)	205.63

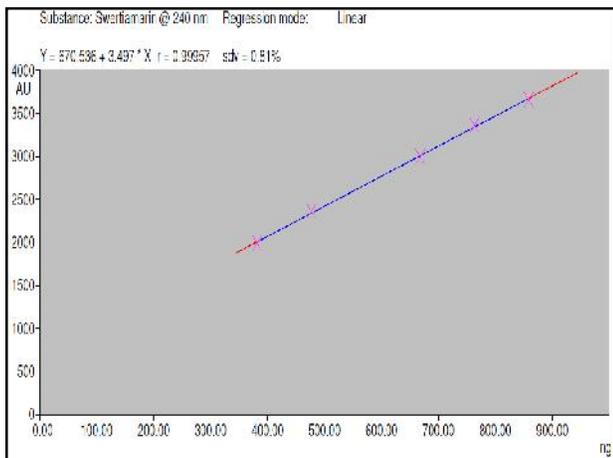


Fig 6: Calibration curve of Standard Swertiamarin (n = 7)

Limit of Detection and Limit of Quantification

The Limit of detection (LOD) under the stated condition estimated for Swertiamarin was 67.8 ng/spot and limit of quantification (LOQ) under the stated experimental conditions obtained was found to be 205.6 ng/spot with good linearity.

Accuracy

By adding known amount of standard analyte in the sample % recovery was measured which was found to be in range from 93.12 to 101.78 % (Table 2). RSD % was found to be in range of 1.30 – 1.82.

Table 2: Percentage Recovery of Swertiamarin

Amount of standard	Theoretical amount (ng)	Analysed amount (ng)	% Recovery	% RSD
31.8	185.8	173.01	93.12	1.82
42.4	196.4	199.9	101.78	1.44
63.6	217.6	205.81	94.58	1.3

Intermediate Precision (Reproducibility)

Precision of the method was as evaluated for intraday and interday analysis of samples. For intraday precision 3 samples were analysed and % RSD calculated was found to be < 2.00 (Table 3). Interday precision performed for 3 different days was estimated to be < 2.00 (Table 4).

Method precision (Repeatability)

10 Sample with same concentration were quantified under same experimental conditions and % RSD was found to be < 2.00 (Table 5).

Table 3: Intraday Precision results

Sample	MKR - 1 (1 µl/spot)			MKR - 2 (1 µl/spot)			MKR - 3 (1 µl/spot)		
	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
Peak Area (AU)	2413.42	2397.97	2438.07	2389.11	2415.41	2354.16	2369.53	2415.26	2323.34
Mean	2416.49			2386.23			2369.38		
Standard	20.23			30.73			45.96		
% RSD	0.84			1.29			1.94		

Table 4: Interday Precision results

Sample	MKR - 1 (1 µl/spot)			MKR - 2 (1 µl/spot)			MKR - 3 (1 µl/spot)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Peak Area (AU)	2416.49	2469.03	2451.97	2386.23	2399.64	2422.06	2369.38	2419.62	2382.61
Mean	2445.83			2402.64			2390.54		
Standard	26.80			18.10			26.04		
% RSD	1.10			0.75			1.09		

Table 5: Method Precision (Repeatability)

Sample	Wt. of sample (gm)	Swertiamarin (mg/tab)
Madhumeha Kusumakar Rasa	0.5349	15.21
	0.5321	15.08
	0.5267	15.08
	0.5002	14.82
	0.5000	14.69
	0.5010	14.82
	0.5114	15.15
	0.5012	14.56
	0.5000	14.43
	0.5123	15.21
Mean		14.91
Standard Deviation		0.2814
% RSD		1.89

Using the developed method for quantification of Swertiamarin by HPTLC, the amount of Swertiamarin in *Madhumeha Kusumakar Rasa* samples MKR-1, MKR-2 and MKR-3 were found to be 15.08 mg/tab, 14.95 mg/tab and 14.30 mg/tab respectively.

DISCUSSION

To develop a HPTLC method, the first crucial step is optimization of Solvent System which facilitates complete extraction of Phyto-constituents. Solvent viz Methanol, Chloroform and Ethyl acetate were took for trials. In this case, Methanol proved to be best suitable extraction solvent.

Selection of appropriate mobile phase is next important aspect in optimization of TLC method to give clear and distinct bands of the various solvent systems experimented. Mobile phase Ethyl acetate: Methanol: Water in the ratio 7.7: 1.3: 0.8 v/v/v expressed distinct bands and showed good resolution between other peaks in densitogram.

The Limit of detection (LOD) which is the concentration of analyte in the sample that can be detected but not necessarily quantified

Results

under the stated condition estimated for Swertiamarin was 67.8 ng/spot and limit of quantification (LOQ) which is the lowest concentration of analyte which can be determined with accuracy under the stated experimental conditions obtained was found to be 205.6 ng/spot with good linearity. This confirmed the sensitivity of developed method to be appropriate.

% RSD reported in Intermediate precision (Intraday and Interday) and method precision indicated the method to be precise.

The accuracy of method developed is also expressed as % recovery estimated after adding known amount of analyte. It is the degree to which the observed results correspond to the true value of the analyte in the sample. The recovery % obtained for Swertiamarin indicated method to be accurate.

CONCLUSION

The proposed method was found to be simple, accurate, precise and specific. This method can be successfully used for quantitative determination of Swertiamarin in *Madhumeha Kusumakar Rasa* and can be applied for routine quality control analysis and authentication of specified Phyto constituents in the formulation.

REFERENCES

1. Maroo J, Vasu VT, Aalinkeel R, Gupta S. Glucose lowering effect of aqueous extract of *Enicostemma littorale* Blume in diabetes: a possible mechanism of action. *J Ethnopharmacol.* 2002 Aug; 81(3):317-20.
2. Sankaranarayanan S, Bama P, Ramachandran J, Kalaichelvan PT, Deccaraman M, Vijayalakshimi M, et al. Ethnobotanical study of medicinal plants used by traditional users in Villupuram district of Ta-

- mil Nadu, India. J Med Plants Res. 2010; 4(12):1089–1101.
- Garad MC, Upadhyaya MA, Kokare DM, Itankar PR. Aerial parts of *Enicostemma littorale* Blume serve as antipyretic and antacid: *in vivo* and *in vitro* evaluations. Pharmacogn Commun. 2012; 2(3) : 42–45.
 - Vaidya H, Rajani M, Sudarsanam V, Padh H, Goyal R. Swertiamarin: A lead from *Enicostemma littorale* Blume for antihyperlipidaemic effect. Eur J Pharmacol. 2009; 617(1–3):108–112. [PubMed]
 - Gite VN, Pokharkar RD, Chopade VV, Takate SB. Hepatoprotective activity of *Enicostemma axillare* in paracetamol induced hepatotoxicity in albino rats. Int J Phar life Sci. 2010; 1(2):50–53.
 - Jaishree V, Badami S, Krishnamurthy PT. Antioxidant effect and hepatoprotective effect of ethyl acetate extract of *Enicostemma axillare* (Lam) Raynal against CCl₄ induced liver injury in rats. Ind J Exp Bio. 2010; 48:896–904. [PubMed]
 - Murali B, Upadhyaya UM, Goyal RK. Effect of chronic treatment with *Enicostemma littorale* in non-insulin dependent diabetic rats. J Ethnopharmacol. 2002; 81:199–204. [PubMed]
 - Upadhyay UM, Goyal RK. Efficacy of *E. littorale* in type 2 diabetic patients. Phyto Res. 2004; 18:233–235. [PubMed]
 - Gohil TA, Patel JK, Vaghasiya JD, Manek Antihyperglycemic and antihyperinsulinemic effect of aqueous extract of *Aegle marmelos* leaf and *Enicostemma littorale*. Ind J Pharm. 2008; 40(2) : 66–91.
 - Bhatt NM, Barua S, Gupta S. Protective effect of *Enicostemma littorale* Blume on rat model of diabetic neuropathy. Am J Infect Dis. 2009; 5(2):106–112.
 - Vishwakarma SL., Bagul MS, Rajni M and Goyal RK, Journal of Planar chromatography A Sensitive HPTLC method for Estimation of Swertiamarin in *Enicostemma littorale* Blume, *Swertia chirata* (Wall) Clarke, and in formulations containing *E. littorale* 17, 128-131. 2004
 - ICH, Q2 (R1), Harmonised Tripartite Guideline, Validation of Analytical Procedures: Text and methodology, International Conference on Harmonization (ICH), Geneva, 2005
 - P. D. Desai, A. K. Ganguly, T. R. Govindachari, B. S. Joshi, V. N. Kamat, A. H. Journal Manmade, S. K. Nagle, R. H. Nayak, A. K. Saksema, S. S. Sathe and N. Vishwanathan, Indian chem. 4, 457-459. 1966
 - Sawant, P. L. Prabhakar B.R., Pandita, N. S., Journal of Planar Chromatography 24 A Validated Quantitative HPTLC method for Analysis of Biomarkers in *Enicostemma littorale* Blume, 6, 497-502. 2011

CORRESPONDING AUTHOR

Dr. K S Thakur

Shree Dhootapapeshwar Limited,
Veer Savarkar Chowk, Panvel,
Navi Mumbai, India

Email: kst@sdindia.com

Source of Support: Nil

Conflict of Interest: None Declared