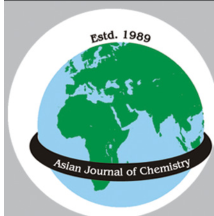
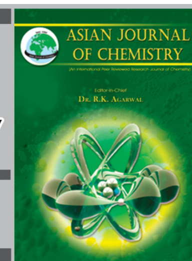


Asian Journal of Chemistry; Vol. 24, No. 10 (2012), 4689-4692

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in


A Validated HPLC Method for the Quantification of Hypericin in *Hypericum perforatum*

A. KAMAL[1], F.J. AHMAD[2,*], S. AHMAD[3] and K. SALEEM[4]

[1]Central Instrumentation Facility, Faculty of Pharmacy, Jamia Hamdard, Hamdard Nagar, New Delhi-110 062, India
[2]Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard, Hamdard Nagar, New Delhi-110 062, India
[3]Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Jamia Hamdard, Hamdard Nagar, New Delhi-110 062, India
[4]Department of Chemistry, Jamia Millia Islamia, Jamia Nagar, New Delhi-110 025, India

*Corresponding author: Fax: +91 11 26059663; E-mail: fjahmad@jamiahamdard.ac.in

(*Received*: 25 August 2011; *Accepted*: 12 May 2012) AJC-11469

A simple, sensitive and specific RP-HPLC method has been developed for the quantification of hypericin in *Hypericum perforatum*. The method involved evaluation of hypericin after resolving it by RP-HPLC with UV detector at 590 nm using acetonitrile:methanol: 10 mM ammonium acetate (pH 5.0) in the ratio 54:36:10 (v/v/v) as mobile phase. The method was validated as per the ICH guidelines for linearity, precision (inter-day, intra-day and inter-system), robustness, accuracy, limit of detection and limit of quantification. The calibration was linear with correlation coefficient of 0.998 over a concentration range of 10-80 μg mL^{-1} for hypericin. The proposed method for the quantification of hypericin was found to be simple, precise, specific, sensitive and accurate and the method can be used for the routine analysis of hypericin for quality control of raw material of *H. perforatum*, several Unani and Ayurvedic formulations containing it as an ingredient.

Key Words: Hypericin, HPLC, Method development, Validation.

INTRODUCTION

Hypericum perforatum L. is a spontaneous perennial herbaceous plant belonging to Guttiferae family. This plant, known as St. John's Wort in Anglo-Saxon folk medicine[1], is widely dispersed in Europe, Asia, northern Africa and north America. The drug Hyperici herba prepared from the dried flowers or dried aerial parts of the plant is known as Erba di S. Giovanni in Italy. Some of the major constituents of *H. perforatum* include napthodianthrone derivatives (0.1-0.3 %), mainly hypericin and pseudohypericin[2]. Other constituents of biological interest are flavones and flavonols, mainly quercitrin, isoquercitrin, rutin and quercetin[2]. Some biflavonoids, *e.g.*, biapigenin derivatives, are also present in the drug[3,4]. *H. perforatum* is largely used as a natural antidepressant. According to the ESCOP monograph[1], the drug is used against mild or moderate depressive states such as restlessness, anxiety and irritability[1]. Recently antiviral and antitumor activities have also been attributed to hypericin and Hyperici herba extracts[5,6]. Hypericin is reported to have monoamine oxidase inhibiting activity. It was found that hypericin in a dose of 0.35 mg has effects similar to imipramine[7] and in a dose of 9-28 μg Kg^{-1} showed activity similar to bupropion[8]. Hypericin has shown to prevent replication of encapsulated viruses *in vivo*[9]. Beside these activities, hypericin has also shown antiinflammatory activity by inhibiting release of leukotrienes[10]. Several analytical methods particularly thin layer chromatography (TLC)[11], UV-VIS spectrophotometry[12], fluorescence spectroscopy[13], non-aqueous capillary electrophoresis[14], cyclic voltametry[15] and high performance liquid chromatography (HPLC)[16] have been reported for determining the hypericin content in the plant samples. The HPTLC, UV-VIS and fluorescence spectroscopic methods are insufficient to determine an accurate amount of hypericins due to interference from other constituents in the extracts. Moreover, the separation of pseudohypericin and hypericin is not good using these methods. The estimation of hypericin, using

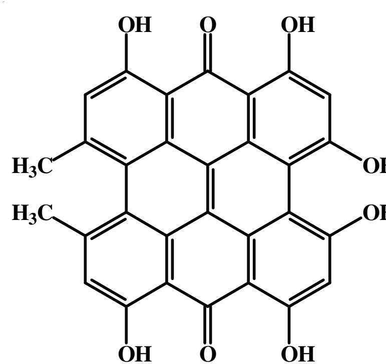


Fig. 1. Chemical structure of hypericin

non-aqueous capillary electrophoresis, cyclic voltametry also showed low resolution owing to poor reproducibility. There are few HPLC methods available for the analysis of hypericins but these methods required lengthy run times and complicated gradient elution systems using solvent mixtures. With this background, we herein report a novel, simple, specific, sensitive and rapid method for the quantitative determination of hypericin using RP-HPLC. This method has been validated as per the ICH guidelines[17].

EXPERIMENTAL

Hypericin standard (98 %) (Batch No. L24285) was procured from Enzo Life Sciences AG, Switzerland. The aerial parts of *Hypericum perforatum* L. were procured from Himalayan Herbs Store, Saharanpur (Uttar-Pradesh), India. All other chemicals were of analytical grade purchased from Merck Specialties Pvt. Ltd., Mumbai (India).

Preparation of standard solutions: Standard solution of hypericin was prepared by dissolving 1 mg of hypericin in 10 mL of methanol (100 μg mL^{-1}). From this stock solution, further dilutions like 10, 20, 40, 60, 80 (μg mL^{-1}) were made to obtain calibration curve of hypericin.

Preparation of sample solutions: Weighed 50 g of dried powdered drug and extracted it with ethanol (80 %, v/v; 250 mL × 3 mL) in a blender with stirring. The hydro alcoholic extract was defatted by liquid liquid extraction with hexane (until colourless) and concentrated to a final amount of 14.65 g dried drug. This sample after suitable dilution with methanol (1 mg mL^{-1}) was filtered through a 0.20 μm non sterile regenerated cellulose membrane (Sartorius AG, Germany).

The HPLC system (Shimadzu Corporation, Japan) consisted of a binary pump (model LC-10AT VP), a UV-VIS detector (model SPD-10AVP), a rheodyne injector (model 77251) equipped with CLASS-VP software (Version 5.032). A reverse phase C_{18} column (Phenomenex, USA, 5 μm, 250 mm × 4.6 mm) attached to a guard column (Shimadzu Corporation Japan, 5 μm, 10 mm × 4.0 mm) was used to separate hypericin. The mobile phase consisted of acetonitrile, methanol, 10 mM ammonium acetate (pH 5.0) in the ratio of 54:36:10, v/v/v). The flow rate of the HPLC system was set at 1.0 mL/min and the run time was 16 min per sample. The applied volume of the sample was 20 μL and hypericin was detected at 590 nm. Water for HPLC was prepared by Millipore instrument (USA).

Linearity: Five point calibration curve was constructed by plotting peak area against concentration. Linearity was evaluated by applying each concentration (10, 20, 40, 60 and 80 μg mL^{-1}) of hypericin in triplicates per sample and five such samples were evaluated (n = 3 × 5).

Method validation

Precision: Precision of a method is the extent to which the individual test results of multiple injections of a series of standards agree. Repeatability was determined by six replicate applications and six times measurement of a standard solution at the analytical concentration of 30, 40 and 60 μg mL^{-1} of hypericin. The repeatability of sample application and measurement of peak area for active compound were expressed in terms of per cent relative standard deviation (% RSD). Precision was obtained from % RSD value by repeating the assay six times on the same day for intra-day precision. Intermediate precision was assessed by the assay of three, six sample sets on different days (inter-day precision) and on different system (inter-system precision). The intra-day, inter-day and inter-system variations for determination of hypericin were carried out at three different concentration levels 30, 40 and 60 μg mL^{-1}.

Robustness of the method: By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions like acetonitrile:methanol:ammonium acetate (pH 5.0) (53.8:36.2:10 and 54.2:35.8:10, v/v/v) were tried and chromatograms were run. The amount of mobile phase was varied in the range of ± 0.2 %. Robustness of the method was done at three different concentration levels 30, 40 and 60 μg mL^{-1}. The wavelengths of the UV-VIS detector were also changed (590 ± 2 nm) and % RSD were determined and found to be less than 2 %.

Limit of detection and limit of quantitation: In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank solution (methanol) was spotted six times following the same method as explained above and the signal to noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting known concentrations of reference solution until the average responses were approximately three or ten times the standard deviation of the responses for six replicate determinations.

Specificity: The specificity of the method was ascertained by analyzing standard drug and sample. The peak for hypericin in sample was confirmed by comparing R_t and the presence of hypericin was also confirmed by comparing UV spectra of sample with that of standard.

Accuracy as recovery: The pre-analyzed samples were spiked with 0, 50, 100 and 150 % of the standard solution and the mixtures were re-analyzed by the proposed method. The experiment was conducted six times. This was done to check the recovery of the drug at different levels in the formulations. Recovery study was carried out for the powdered sample of *H. perforatum*.

RESULTS AND DISCUSSION

Optimization of solvent system: For the development of mobile phase, different trials were made using many solvents in different proportions. When mobile phase consisting of acetonitrile:methanol:10 mM ammonium acetate (pH 5.0) was used in the ratio of 48:32:10 (v/v/v), a peak was observed at the R_t of 7.43 min for hypericin. But it was found that the resolution of the peak was poor. In order to improve the resolution of the peak a new mobile phase with the composition of acetonitrile:methanol:10 mM ammonium acetate was used in the ratio of 54:36:10 (v/v/v). This new mobile phase helped in achieving very sharp peak at the R_t of 7.43 min for hypericin with good resolution of more than one.

Linearity: Linearity was found to be in the concentration range of 10-80 μg mL^{-1} for hypericin with r^2 value of 0.998. This value of correlation coefficient indicated a high degree of linearity (Fig. 5).

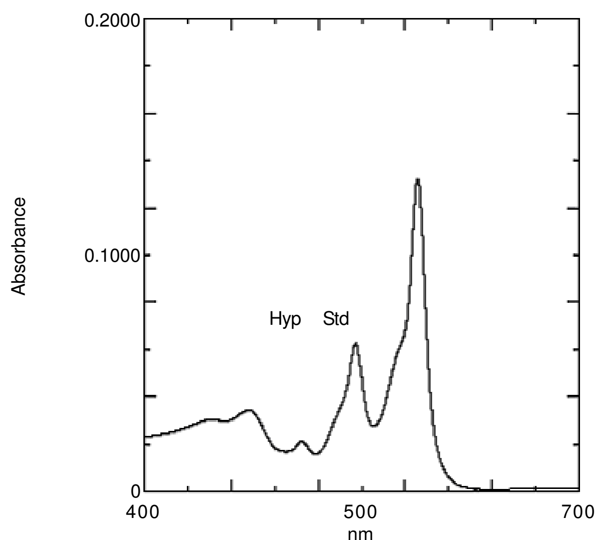


Fig. 2. UV spectrum of hypericin standard (40 µg mL^{-1}) in methanol at 590 nm

TABLE-1
VALIDATION PARAMETERS OF THE PROPOSED HPLC METHOD FOR ESTIMATION OF HYPERICIN

Validation parameters	Results
Linearity range (µg mL^{-1})	10-80
Correlation coefficient (r^2 ± SD)	0.998 ± 0.001
Regression equation	y = 57919x - 36945
Limit of detection (µg mL^{-1})	3.1
Limit of quantification (µg mL^{-1})	9.6

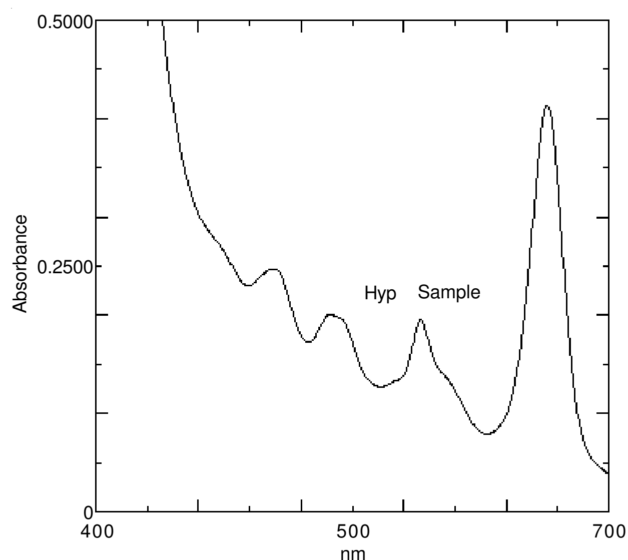


Fig. 3. UV spectrum of hypericin sample in methanol at 590 nm

Method validation

Precision: Precision data on the intra-day, inter-day and inter-system variations for three different concentration levels are summarized in Table-2. The low % RSD indicated that the method is precise for the analysis.

Robustness of the method: The effect of deliberate changes in the composition of mobile phase were studied as % RSD and depicted in Table-3. The low % RSD indicated that the method is robust.

Limit of detection and limit of quantitation: For the proposed method LOD and LOQ were calculated using signal to noise ratio method and found to be 3.1 and 9.6 µg mL^{-1} for hypericin (Table-1).

Specificity: The specificity of the newly proposed method was ascertained by superimposing the UV spectrum of both standard and sample and confirmed for its purity (Fig. 4).

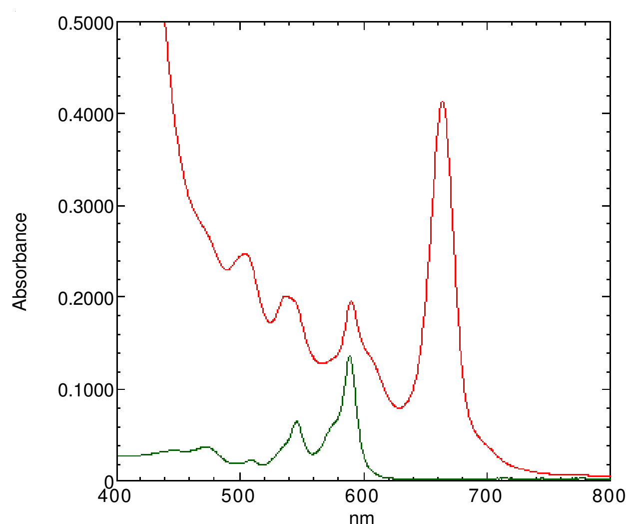


Fig. 4. Overlay UV spectra of hypericin standard with sample at 590 nm

Recovery studies (accuracy): The accuracy for the method was done as recovery studies and the amount of the drug recovered was calculated on the basis of % RSD. The results of the recovery study were depicted in Table-4.

Analysis of samples: For the analysis, samples were applied in triplicates; hypericin has R_t of 7.43 min (Figs. 6 and 7). It was found that no interference is there in samples with immediate impurities and resolution between the peaks was good.

Conclusion

A HPLC method was developed and validated for the determination of hypericin and the content of this marker

TABLE-2
INTERMEDIATE PRECISION OF THE PROPOSED HPLC METHOD OF HYPERICIN

Conc. (µg mL^{-1})	Inter-day precision		Intra-day precision		Inter-system precision	
	Mean peak area ± SD (n = 6)	RSD (%)	Mean peak area ± SD (n = 6)	RSD (%)	Mean peak area ± SD (n = 6)	RSD (%)
30	1073365 ± 9150.1	0.85	1053790 ± 5109.5	0.48	1059927 ± 5518.0	0.52
40	1947935.5 ± 8600.3	0.44	1990292 ± 7280.7	0.36	1994013.3 ± 16029	0.80
60	3027152 ± 22317	0.73	3000769 ± 40534	1.35	3031723.6 ± 16972	0.55

RSD: Relative standard deviation.

TABLE-3
ROBUSTNESS OF THE PROPOSED HPLC METHOD OF HYPERICIN

Mobile phase composition change (acetonitrile:methanol:ammonium acetate)			Mean area ± SD (n = 3)	RSD (%) of area
Actual (v/v/v)	Used (v/v/v)	Level		
54:36:10	53.8: 36.2: 10	-0.2	1876919.3±20285	1.08
	54: 36: 10	0	1843475.6±13971	0.75
	54.2: 35.8: 10	+0.2	1862811.6±14179	0.76
Wavelength change				
Actual (nm)	Used (nm)	Level		
590	588	-2	1904265 ± 13988	0.73
	590	0	1887467.3 ± 20893	1.10
	592	+2	1896115 ± 20910	1.10

RSD: Relative standard deviation.

TABLE-4
ACCURACY AS RECOVERY OF THE PROPOSED HPLC METHOD OF HYPERICIN

Percentage of standard spiked to the sample	Theoretical content (µg mL^{-1})	Amount of drug recovered (µg mL^{-1}) ± SD) (n = 6)	Percentage of drug recovered	RSD (%)
0	2800	2814.2 ± 7.1	100.50	0.25
50	4200	4204.1 ± 27.6	100.09	0.65
100	5600	5586.9 ± 17.0	99.76	0.30
150	7000	7014.2 ± 50.4	100.20	0.71

RSD: Relative standard deviation.

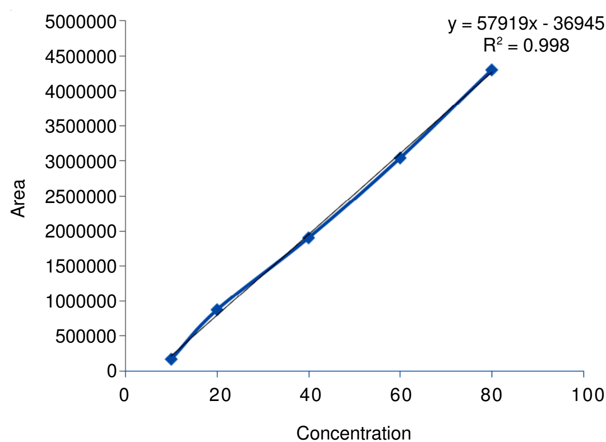


Fig. 5. Calibration curve of hypericin standard

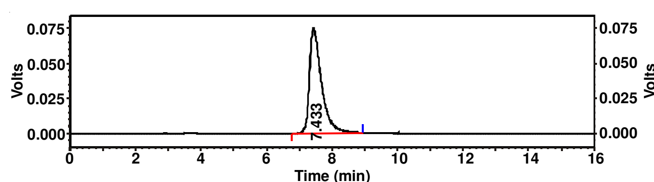


Fig. 6. HPLC chromatogram of hypericin standard (40 µg mL^{-1}) at 590 nm

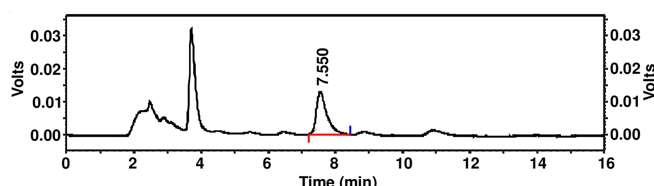


Fig. 7. HPLC chromatogram of hypericin sample at 590 nm

present in *H. perforatum* plant was quantified and found to be 0.28 % w/v for hypericin. The method was found to be simple, rapid, accurate, specific and robust for the analysis of hypericin in crude drug and can be adopted by any laboratory for the quality control of crude drugs and formulations that contains hypericin as an active marker.

REFERENCES

1. ESCOP Monographs, Hyperici Herba, St. John's Wort, Copyright ESCOP, March, pp. 1-10 (1996).
2. R.D. Loggia, In Piante officinali per infuse e tisane, OEMF, Milano, p. 274 (1993).
3. R. Berghofer and J. Holzl, *Planta Med*., **53**, 216 (1987).
4. R. Berghofer and J. Holzl, *Planta Med*., **55**, 91 (1989).
5. G. Lavie, Y. Mazur, D. Lavie, A.M. Pruice, D. Pascual, L. Liebes, B. Lavin and D. Meruelo, *Transfusion*, **35**, 392 (1995).
6. P.A. Cohen, J.B. Hudson and G.H. Towers, *Experimentia*, **15**, 180 (1996).
7. R.B. Raffa, *Life. Sci*., **62**, 265 (1998).
8. A. Nahrstedt and V. Butterweck, *Pharmacopsyciatry*, **30**, 129 (1997).
9. D. Meruelo, D. Lavie and E. Lavie, *Proc. Natl. Acad. Sci. USA*, **85**, 5230 (1989).
10. A.G. Panossian, E. Gabrielian, V. Manvelian, K. Jurcic and H. Wagner *Phytomedicine*, **3**, 19 (1996).
11. N. Mulinacci, C. Bardazzi, A. Romani, P. Pinelli, F.F. Vincieri and A. Constantini, *Chromatographia*, **49**, 197 (1999).
12. J. Zhao, W. Meng, P. Miao, Z. Yu and G. Li, *Int. J. Mol. Sci*., **9**, 145 (2008).
13. S. Kascakovas, M. Refregiers, D. Jancura, F. Sureau, J.C. Maurizot and P. Miskovsky, *Photochem. Photobiol*., **81**, 1395 (2005).
14. A.G. Jensen and S.H. Hansen, *J. Pharm. Biomed. Anal*., **27**, 167 (2002).
15. J. Redepenning and N. Tao, *Photochem. Photobiol*., **58**, 532 (1993).
16. T. Sirvent and D.M. Gibson, *J. Liq. Chromatogr. Rel. Technol*., **23**, 251 (2000).
17. Q2A, ICH, Q2A (R1), Validation of Analytical Procedures: Text and Methodology, International Conference on Harmonization, Nov. (2005).