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Development and validation of HPLC-UV method for estimation of swertiamarin in *Enicostemma littorale*

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Abstract

Secoiridoid glycosides are a group of phytoconstituents present in Gentianaceae family and are known for their antidiabetic and hypolipedimic activities. Swertiamarin is the major physiologically active secoiridoid glycoside reported in *Enicostemma littorale* Blume (Gentianaceae). This study is designed for development and validation of an HPLC-UV method for estimation of swertiamarin. The developed HPLC-UV method was found accurate and reproducible for the detection and quantification of swertiamarin. A well-resolved peak of swertiamarin appeared at Rt 3.51±0.07 min with methanol-water (80:20) as mobile phase. The linear regression analysis data for the calibration curve showed good linearity in the concentration range of 100-1000 µg/ml with a correlation coefficient (r2) of 0.9994. The linear regression equation was y=6310.5x+869205. The limit of detection and limit of quantitation were found to be about ≈19 and ≈58 µg/ml, respectively. The method was found to be accurate (98.09-100.70 % recovery), precise (< 2 % R.S.D.) and robust (1.5 % R.S.D.). The proposed method was employed for the estimation of swertiamarin *in E. littorale*. Statistical analysis proved that the method was precise, selective and accurate for the estimation of swertiamarin.

Keywords: Swertiamarin, *Enicostemma littorale*, Gentianaceae, HPLC, ICH guidelines

INTRODUCTION

Enicostemma littorale Blume (Gentianaceae) locally known as Chota-chiretta, is commonly used Ayurvedic medicine for the treatment of diabetes. It is a glabrous perennial herb, distributed throughout India and known for several medicinal uses like antidiabetic, hypolipidemic and antipyretic (Nadkarni, 2007). Swertiamarin (Figure 1) is a secoiridoid glycoside distributed among the members of Gentianaceae. Swertiamarin is reported to have antihyperlipidaemic (Vaidya et al., 2009a and 2009b), hypoglycemic (Patel and Mishra, 2011), insulinotropic (Sonawane et al., 2010; Maroo et al., 2002; Vaidya et al., 2012) and antinociceptive (Jaishree et al., 2009) activities. As sample preparation is a fundamental and critical step with important consequences for the accuracy of results, ultrasound-assisted extraction (UAE) recently becomes useful tool for extraction of plant material as it is very simple, rapid and has no limitations regarding extraction solvent choice. UAE utilizes sonic energy to assist the solvent extraction of phytoconstituents (Kamaljit et al., 2008). UAE has been applied for the extraction of phytosterols (Melecchi et al., 2006), flavonoids (Pan et al., 2012), polysaccharides (Chen et al., 2012) and antioxidants (Gribova et al., 2008) from plants. A through review of literature has revealed that few analytical methods have been reported for the estimation of swertiamarin in plants, extracts and formulations (Alam et al., 2009 and 2011). Neither of these methods are sensitive or accurate for the analysis of swertiamarin. The purpose of this research was to establish and validate, in accordance with International Conference on Harmonization (ICH) guidelines, a simple, accurate, economical and

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reproducible HPLC-UV method for estimation of swertiamarin in extracts of *E. littorale*.

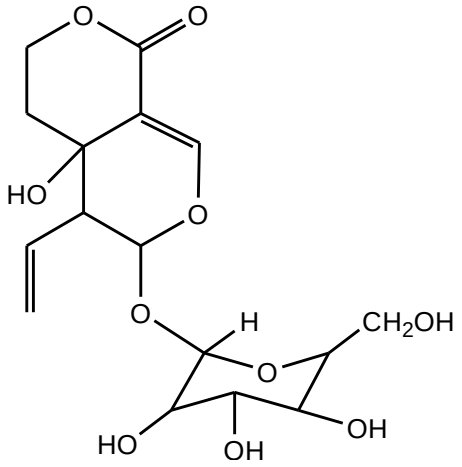

Figure 1. Molecular structure of swertiamarin

MATERIALS AND METHODS

Plant material and chemicals

The whole plant of *E. littorale* was procured from local crude drug market, Delhi, and identified by Dr. H. B. Singh, Chief Scientist (RHMD), NISCAIR, New Delhi, India. A voucher specimen of the sample (NISCAIR/RHMAD/Consult/-2011-12/1856/01) was deposited in the RHM Division, NISCAIR, New Delhi. Standard swertiamarin (purity ≥ 98.24 %) was purchased from Shenzhen Sungening Bio-Tech., China. HPLC grade solvents and reagents were obtained from S.D. Fine Chemicals, Mumbai, India. All other chemicals were obtained from Merck, Mumbai, India and were of analytical grade.

Sample preparation

E. littorale (whole plant) was cleaned and dried in an oven at 45 °C. Dried sample was pulverized to a coarse powder using a grinder. About 10 g of powder was placed in a stoppered conical flask and extracted with 250 ml solvent consisting of methanol-water mixture (80:20 % v/v), at 50 °C for 50 min in an ultra-sonicator (Toshniwal, India) at 200 W ultrasonic power. The extract was filtered and evaporated under reduced pressure at 50 °C in a rotary evaporator (Buchi, Switzerland). The residue was then reconstituted in 10 ml methanol and filtered through 0.45 µm membrane filter and stored at 4 °C for further analysis.

Preparation of standard solution and quality control (QC) samples

Accurately weighed 10 mg of swertiamarin reference standard and transferred to 10 ml volumetric flask. Methanol was added and sonicated in ultrasonic water bath and final volume made up to 10 ml, this gives concentration of 1 mg/ml and used as standard stock solution. Quality control (QC) samples at three different concentration levels (200, 400 and 600 µg/ml arbitrary units as low, medium and high) were prepared independent of the calibration standards.

HPLC instrumentation and conditions

A HPLC system (Shimadzu) with quaternary LC-10A VP pumps, variable wavelength programmable UV/VIS detector, SPD-10AVP column oven (Shimadzu) and SCL 10 AVP system controller (Shimadzu). 25 µl syringe (Hamilton Co., RENO, NEVADA) was used to inject samples. The chromatographic column used was a reverse phase C18, 250 X 4.6 mm, 5 µm, Zorax RP-HPLC. The column and HPLC system were kept at ambient conditions.

Method development

Various solvent mixtures were tried for the development of a suitable mobile phase. Methanol-water (50:50, 60:40, 70:30 and 80:20 % *v/v)* and acetonitrile-water (10:90, 40:60 and 90:10 % *v/v*) were tried as mobile phase. The suitability of the

solvent mixtures was decided by parameters such as peak separation, shape, sensitivity of the assay, cost and the time required for analysis. The mobile phase was injected at 1 ml/min and elutes were analyzed at various wavelength. The stability of analyte in solution during analysis was also determined by repeated analysis of samples during the course of experimentation on the same day and also after 48 h storage of the solution at laboratory conditions and in the refrigerator.

Preparation of calibration plots

Various concentrations ranging from 100-1000 µg/ml were made from the standard stock solution and were analysed in triplicate. Linearity curve plotted between mean area and concentration and was treated by linear least-square regression analysis.

Method Validation

The developed method was validated as per ICH guidelines by determining linearity range, precision, accuracy, robustness, limits of detection (LOD), limit of quantification (LOQ) and recovery similar to method reported by laboratories for quality control of different drugs (ICH guidelines, 2005; Ahamad et al., 2014; Jain et al., 2013; Khan et al., 2013).

Precision

The precision and accuracy of the system was determined by measuring repeatability of sample application and measurement of concentration for six replicates of the bands (200, 400, 600 µg/ml). Intra and inter-day variation for the determination of drugs 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 standard error of mean (S.E.M.).

Accuracy

Accuracy was determined as percent recovery by the standard addition method. The pre-analysed samples of swertiamarin (100 µg/ml) were spiked with 50, 100 and 150 % of the standard and the mixtures were reanalysed in triplicate by the developed assay. Percent recovery and % R.S.D. were calculated at each concentration level.

Robustness

Robustness of the method was determined by changing the flow rate (0.9 to 1.1 ml/min), mobile phase composition (methanol-water, 78:22 to 82:18 *% v/v*) and run time (8 to 12 min) of assay procedure. The change in retention time (R_t) was recorded and % R.S.D. was calculated.

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were determined by standard deviation (SD) method. LOD and LOQ were determined by injecting six blank samples, peak areas were recorded. LOD and LOQ were determined using the slope of the calibration curve and S.D. of the blank sample.

Specificity

The specificity of method was ascertained by analyzing standard and test samples. The band for swertiamarin in test samples was confirmed by comparing R_f and UV spectra of band with that of standard. The peak purity of swertiamarin was assessed by comparing the spectra at three different levels *i.e.* peak start, peak apex and peak end position of the band.

Application of method

The test samples were injected and chromatograms were obtained under the same conditions as that of standard swertiamarin. The peak area of the peak corresponding to R_t of standard swertiamarin was recorded and content of the same was calculated from the regression equation obtained from calibration curve.

RESULTS AND DISCUSSION

Chromatographic conditions

An HPLC method for quantification of swertiamarin in plants, and formulation has been reported earlier (Alam et al., 2009) with a total run time of 30 min. Number of mobile phases were tried but they resulted either in poor resolution, base line problem, long R_t or trailing. The chromatogram obtained with methanol-water (80:20 % *v/v*) solvent system has lowest R_t (3.51 min) and sharp well defined peak (Figure 2). Therefore the mixture of methanol-water (80:20 % *v/v*) was selected as the mobile phase. The drug was stable for a period of 48 h storage at laboratory temperature and under refrigerator temperature in mobile phase.

Calibration curve

The calibration curve area versus concentration (µg/ml) was found linear in the range of 100-1000 µg/ml. The linear regression data for the calibration curve showed a good linear relationship over the concentration ranges of 100-1000 µg/ml with respect to peak area. The retention time was found to be 3.51±0.07 min.

Validation of the developed assay

Linearity

The linearity range of swertiamarin was obtained as 100-1000 µg/ml as shown in Table 1. The regression equation was Y = 6310.5X + 869205 with correlation coefficient (r^2) of 0.9994.

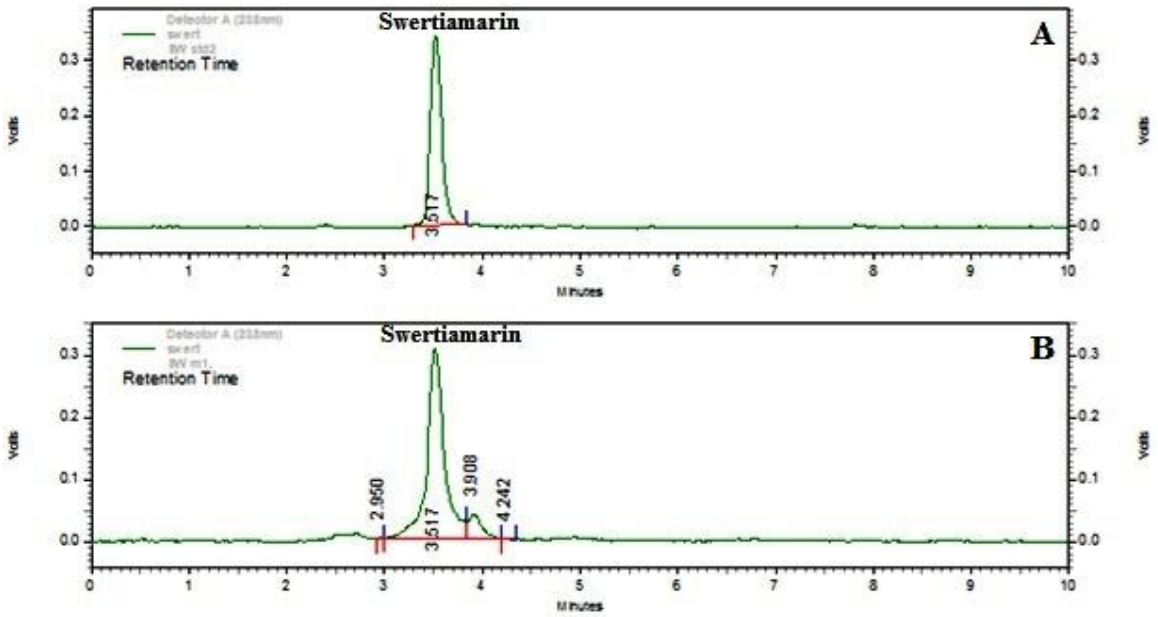

Figure 2. HPLC Chromatogram (**A**) standard swertiamarin showing R_t at 3.517 min with methanol-water (80:20) (**B**) HPLC Chromatogram *Enicostemma littorale* extract showing R_t at 3.517 min with methanol-water (80:20).

Table 1: Linear regression data for the calibration curve

Parameters	Swertiamarin
Linearity range (µg/ml)	100 - 1000
Regression equation	y = 6310.5 x + 869205
Correlation coefficient (r^2)	0.9994
LOD (µg/ml)	19.16
LOQ (µg/ml)	58.06

n=3, S.D.= Standard deviation

Precision

Results of repeatability and intermediate precision are shown in Table 2. The low values of % R.S.D. indicated the high degree of repeatability and intermediate precision of the proposed method.

recovery of 98.09-100.70 % after spiking the additional standard solution to the previously analysed solution. The values of percent recovery and % R.S.D. are shown in Table 3 that indicated high degree of accuracy of the assay method.

Robustness

Robustness was determined to evaluate the influence of small but deliberate variation in the chromatographic conditions in the assay procedure. There was no significant change in the R_t of swertiamarin by changing the flow rate, mobile phase composition and run time. Low value of the % R.S.D., indicated the robustness of the method as shown in Table 4.

Table 2: Precision of swertiamarin assay method

Conc. (µg/ml)	Repeatability (intraday precision)			Intermediate precision (interday)		
	Mean area ± S.D.	S.E.M.	% R.S.D.	Mean area ± S.D.	S.E.M.	% R.S.D.
200	2166825 ± 6622	3823.7	0.30	2125497 ± 7158	4132.8	0.33
400	3482490 ± 6737	3890.1	0.19	3463223 ± 42514	24546	1.22
600	4676510 ± 9419	5438.4	0.20	4661934 ± 39735	22941	0.85

n=3, S.D.= standard deviation, S.E.M.= standard error of mean, R.S.D.= relative standard deviation

Table 3: Accuracy of swertiamarin assay method

Excess analyte added (%)	Theoretical content (µg)	Concentration found (µg) ± S.D.	Recovery (%)	% R.S.D.
0	100	98.09 ± 1.98	98.09	1.35
50	150	152.06 ± 3.84	101.37	1.13
100	200	199.11 ± 1.61	99.55	0.81
150	250	251.76 ± 6.37	100.70	1.62

n=3, S.D.= standard deviation, R.S.D.= relative standard deviation

Table 4: Robustness of swertiamarin assay method

#Parameters	Mean area ± S.D.	% R.S.D.
Flow rate (0.9 to 1.0, ml/min)	2157579 ± 16014	0.74
Mobile phase composition (± 2 ml)	2145388 ± 21476	1.00
Run time (8 to 12 min)	2137056 ± 3957	0.18

 # for 200 µg/ml, n=3, S.D.= standard deviation, R.S.D.= relative standard deviation

Accuracy

The accuracy of the proposed method was calculated by recovery analysis, which afforded the

Limit of detection and limit of quantitation

LOD and LOQ of the proposed method were determined by the standard deviation method and

were found to be 19.16 and 58.06 µg/ml respectively, which indicated that the proposed method could be used in wide range for detection and quantification of swertiamarin effectively.

Specificity

The specificity of proposed method was determined by comparing the sample and standard peak for its R_t and UV spectra. Three point peak purity *i.e.* peak start, peak apex and peak end, was compared and was found superimposed (Figure 3). This indicated that the standard and sample peaks were not merging with any other components or impurities (Figure 2B).

Application of method

The proposed method was successfully applied for the detection and quantification of swertiamarin in different plant samples. The content of swertiamarin in hydro-alcoholic extract of *E. littorale* was found to be 12.62±2.18 % on dry weight basis of plant material.

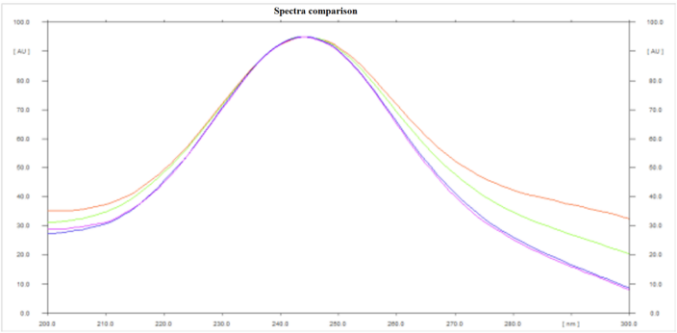

Figure 3. UV overlay spectra of swertiamarin in standard and test samples

Conclusion

An HPLC method has been developed for identification and quantification of swertiamarin. Low cost, faster speed and satisfactory precision (R.S.D. <2%), and accuracy are the main features of this method. The method was successfully validated as per the ICH guidelines, and statistical data prove that the method is sensitive, specific, precise and repeatable. It is used as a analytical tool for the routine analysis of this antidiabetic compound.

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References

1. Ahmad, J., Amin, S., Mir, S.R., 2014. Development and validation of HPTLC densitometric method for estimation of charantin in Momordica charantia fruits and herbal formulation. Journal Pharmacognosy and Phytochemistry. 5, 2126-130.
2. Alam, P., Ali, M., Singh, R., Shakeel, F., 2009. Estimation of swertiamarin in *Enicostemma littorale* and marketed formulations using HPLC-UV Method. Journal of Bioanalysis and Biomedicine. 1(1), 22-27.
3. Alam, P., Ali, M., Singh, R., Shakeel, F., 2011. A new HPTLC densitometric method for analysis of swertiamarin in *Enicostemma littorale* and commercial formulations. Natural Product Research. 25(1), 17-25
4. Chen, W., Wang, W.P., Zhang, H.S., Huang, Q., 2012. Optimization of ultrasonic-assisted extraction of water-soluble polysaccharides

from Boletus edulis mycelia using response surface methodology. Carbohydrate Polymer. 87(1), 614-619.

5. Gopal, R., Gnanamani, A., Udayakumar, R., Sadulla, S., 2004. *Enicostemma littorale* Blume: A potential hypolipidemic plant. Natural Product Radiance. 3, 401-405.
6. Gribova, N.Y., Filippenko, T.A., Nikolaevskii, A.N., Belaya, N.I., Tsybulenko, A.A., 2008. Optimization of conditions for the extraction of antioxidants from solid parts of medicinal plants. Journal of Analytical Chemistry. 63(11), 1034-1037.
7. ICH. 2005. Validation of analytical procedures: Text and Methodology. ICH-Q2 (R1), International Conference on Harmonization: Geneva.
8. Jain, P., Girase, D., Bedse, A., Surana, S., 2013. Stability-Indicating HPTLC densitometric method for determination of Metformin hydrochloride in tablet formulation. Journal of pharmaceutical and Biosciences. 1(2): 51-58.
9. Jaishree, V., Badami, S., Kumar, R.M., Tamizhmani, T., 2009. Antinociceptive activity of swertiamarin isolated from *Enicostemma littorale* . Phytomedicine, 16, 227-232.
10. Kamaljit, V., Raymond, M., Lloyd, S., Darren, B., 2008. Application and opportunities for ultrasound assisted extraction in the food industry: a review. Innovative Food Science and Emerging Technology. 9(2), 161-169.
11. Kavimani, S., Manisenthlkumar, K.T., 2000. Effects of methanolic extract of *Enicostemma littorale* on Dalton's ascetic lymphoma. Journal of Ethnopharmacology. 71, 349-352.
12. Khan Md. M.G., Jain, P.S., Shirkhedkar, A.A., Fursule, R.A., Kale, N.K., Surana, S.J. 2013. Stability indicating HPLC determination of Erdosteine in bulk drug and pharmaceutical dosage form. Journal of Pharmaceutical and Biosciences. 1(3), 105-109.
13. Maroo, J., Vasu, V.T., Aalinkeel, R., Gupta, S., 2002. Glucose lowering effect of aqueous extract of *Enicostemma littorale* Blume in diabetes: a possible mechanism of action. Journal of Ethnopharmacology. 81(3), 317-320.
14. Melecchi, M.I., Peres, V.F., Dariva, C., Zini, C.A., Abad, F.C., Martinez, M.M., Caramao, E.B., 2006. Optimization of the sonication extraction method of Hibiscus tiliaceus L. flowers. Ultrasonic Sonochemistry. 13(3), 242-250.
15. Nadkarni, K.M., 2007. Indian Materia Medica, volume 1, Popular Prakashan Pvt. Ltd. Mumbai, pp. 185.
16. Pan, G.Y., Yu, G.Y., Zhu, C.H., Qiao, J.L., 2012. Optimization of ultrasound-assisted extraction (UAE) of flavonoids compounds (FC) from hawthorn seed (HS). Ultrasonic Sonochemistry. 19(3), 486-490.
17. Patel, M.B., Mishra, S.H., 2011. Hypoglycemic

activity of C-glycosyl flavonoid from Enicostemma hyssopifolium. Pharmaceutical Biology. 49(4), 383-391.

18. Sadique, J., Chandra, T., Thenmozhi, V., Elango, V., 1987. The anti-inflammatory effects of *Enicostemma littorale* and Mollugo cerviana. Biochemical Medicine and Metabolic Biology. 37, 167-176.
19. Sonawane, R.D., Vishwakarma, S.L., Lakshmi, S., Rajani, M., Padh, H., Goyal, R.K., 2010. Amelioration of STZ-induced type 1 diabetic nephropathy by aqueous extract of *Enicostemma littorale* Blume and swertiamarin in rats. Molecular and Cellular Biochemistry. 340, 1-6.
20. Vaidya, H., Goyal, R.K., Cheema, S.K., 2012. Anti-diabetic activity of swertiamarin is due to an active metabolite, gentianine, that upregulates PPAR-g gene expression in 3T3-L1 cells. Phytotherapy Research. DOI: 10.1002/ptr.4763.
21. Vaidya, H., Rajani, M., Sudarsanam, V., Padh, H., Goyal, R., 2009a. Antihyperlipidaemic activity of swertiamarin, a secoiridoid glycoside in poloxamer-407-induced hyperlipidaemic rats. Journal of Natural Medicine. 63, 437-442.
22. Vaidya, H., Rajani, M., Sudarsanam, V., Padh, H., Goyal, R., 2009b. Swertiamarin: a lead from *Enicostemma littorale* Blume for anti-hyperlipidaemic effect. European Journal of Pharmacology. 617(1-3), 108-112.