Abstract
A simple, economic, reproducible, robust and precise HPLC method for estimation of swertiamarin in both 60% methanolic extract of *Enicostemma littorale* and marketed formulations was developed and validated in present investigation. The mobile phase composed of methanol and water (90:10 % v/v), gave a sharp and well-defined peak of swertiamarin at the retention time of 10.15 ± 1.52 min. The limit of detection (LOD) and limit of quantification (LOQ) were 17.25 and 56.92 µg/ml respectively. The proposed method with high degree of precision and accuracy was employed for the estimation of swertiamarin in methanolic extract and in marketed formulation.

Keywords: Swertiamarin; HPLC; ICH guidelines; *Enicostemma littorale*

Introduction
*Swertia* Linn. (Gentianaceae) is an annual or perennial herb comprising more than 170 species among which 79 and 40 species are distributed in China and India respectively (Bhandari et al., 2006a). Approximately 20 species of this genus are used in Chinese traditional medicine to treat hepatic, choleric and anti-inflammatory diseases (Bhandari et al., 2006a). *Enicostemma littorale* (Chota-Kirayat or Chota-Chirayata) is a glabrous perennial herb belongs to family Gentianaceae (Murali et al., 2003). It is a perennial herb attaining height of 15-20 inch with sessile lanceolate leaves and is found throughout India up to a height of 1500 ft (Bhandari et al., 2006a). This plant has been widely used in traditional system of medicines for the treatment of diabetes, malaria and fewer (Maroo et al., 2003). This herb is also known for its anti-inflammatory effects (Sadique et al., 1987), antican-cer activity (Kavimani and Manisenthilkumar, 2000), hypoglycemic, antioxidant and hypolipidemic potential in newly-diagnosed non-insulin-dependent diabetes mellitus (NIDDM) patients (Maroo et al., 2003). Its antidiabetic effects were also reported by other workers (Murali et al., 2002; Upadhyay and Goyal, 2004; Srinivasan et al., 2005). It is used as active ingredient in an antidiabetic herbomineral preparation (Murali et al., 2002; Babu and Prince, 2004). Recently aerial part of *E. littorale* was reported to show hypolipidemic effect in *p*-dimethylaminobenzenzine (PDAB) induced hepatotoxic animals (Gopal et al., 2004). A through literature survey has revealed that few analytical methods have been reported for the estimation of swertiamarin in plant, its extract and in formulations (Takie et al., 2001; Vishwakarama et al., 2004; Bhandari et al., 2006a; Bhandari et al., 2006b). Only two HPTLC methods are available for estimation of swertiamarin in *E. littorale* and its formulations (Vishwakarama et al., 2004; Bhandari et al., 2006b). Therefore the aim of present study was to develop a simple, economical, selective, precise, reproducible and robust high performance liquid chromatographic (HPLC) method for the estimation of swertiamarin in *E. littorale*, its extracts and in marketed formulation using UV detector. The proposed method was validated according to ICH guidelines (ICH, 1996).

Experimental

Plant material and chemicals
*Enicostemma littorale* (whole plant) was collected from three different geographical regions viz- Shivapuri (M.P), Ajmer (RAJ) in the month of February whereas sample from Haridwar (UA) was collected in the month of March. These plants were assigned batches like EL/SGTC/MP, EL/SSH/RAJ and EL/SSH/UA respectively in the samples obtained from M.P, Rajasthan and Uttaranchal (India). The identity of these batches was confirmed by taxonomist of Ranbaxy Research Laboratories, Gurgaon, India. The voucher specimen was deposited in Phytochemistry research laboratory, Jamia Hamdard (New Delhi), India. All the solvents were of HPLC grade and other chemicals used were of analytical reagent (AR) grade and obtained from E-Merck, Mumbai, India.

Marketed formulations
Two traditional ayurvedic marketed formulations A (tablet) and B (capsule) containing *E. littorale* extract was purchased from local market and used for quantification of swertiamarin.

Preparation of sample solution
About 5 g of the crude drugs weighed and refluxed with 100 ml of methanol for 1 h in water bath and filtered through whatmann filter paper (No. 41). The marc left out was refluxed again with 50 ml of methanol for 1 h and filtered. The filtrates were combined and concentrated to 25 ml in rotary vacuum evaporator (Medica Instrument Mfg. Co, Mumbai, India) to a
final volume of 25 ml. This solution was used as test solution in the HPLC analysis.

Sample preparation for quantification of swertiamarin in marketed formulations

6 tablets (formulation A) or 6 capsules (formulation B) were crushed and dissolved in 50 ml of distilled water, transferred to a separating funnel. It was then extracted thrice with 140 ml each of extraction media (chloroform: methanol: butanol-25:2:3). The organic layer was passed over anhydrous sodium sulphate and evaporated to dryness on water bath under vacuum. The residue left was reconstituted in 20 ml of methanol, which was used as test solution in HPLC analysis.

Preparation of standard solution

Accurately weighed 10.2 mg of swertiamarin (purity 98 %) reference standard was transferred to 25 ml volumetric flask. Methanol was added and sonicated in ultrasonic water bath to dissolve. Volume was made up with methanol to 25 ml. This gives concentration of 399.84 µg/ml. 10 ml of this solution was further diluted to 13.32 ml with methanol to get concentration of 300 µg/ml. This solution was used as a reference solution (stock solution) for swertiamarin.

Equipment and conditions

A Shimadzu model HPLC equipped with quaternary LC-10A VP pumps, variable wavelength programmable UV/VIS detector SPD-10AVP column oven (Shimadzu), SCL 10AVP system controller (Shimadzu), Rheodyne injector fitted with a 20 µl loop and Class-VP 5.032 software was used. The chromatographic column used was a reverse phase C18, 250 x 4.6mm, 5micZorax RP-HPLC. The column and HPLC system were kept at ambient conditions. The mobile phase was Methanol: water (9:1) with the flow rate of 1.0 ml/min. The injection volume was 20 µl and the elutes were analyzed at a wavelength of 338 nm.

Method development

Various solvent systems were tried for the development of a suitable HPLC method for estimation of swertiamarin in 60% methanolic extract and marketed formulations. Mobile phases tried for these purposes were methanol: water (10:90 %), methanol: water (40:60 %), methanol: water (90:10 %), acetonitrile: water (10:90 %) acetonitrile: water (40:60 %) and acetonitrile: acetonitrile: water (90:10 %). The suitability of the solvent system was decided by cost, sensitivity of the assay and time required for the analysis.

Calibration curve of swertiamarin

Various concentrations (50-300 µg/ml) were made for the preparation of calibration curve from the prepared standard stock solution. The mobile phase after filtration through 0.45 µm membrane filter was delivered at 1.0 ml/min for column standardization. The baseline was continuously monitored during the column standardization. The wavelength of detection was selected at 338 nm. The prepared dilutions (concentrations) were injected serially and areas under the peaks were recorded for each concentration. The stability of drug 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 drug solution at laboratory conditions and in the refrigerator.

Method validation

Linearity

The linearity of swertiamarin was checked between 50-300 µg/ml concentration range. Graph was plotted between concentration and area for linearity.

Accuracy as recovery

Accuracy was determined as % recovery by standard addition method. The preanalyzed sample of swertiamarin (100 µg/ml) was spiked with the extra 0, 50, 100 and 150 % of the standard swertiamarin and the mixtures were reanalyzed by the proposed method. The % recovery and percent relative standard deviation (% RSD) were calculated at each concentration level.

Precision

Precision was determined at two levels according to ICH guidelines (repeatability and intermediate precision). Repeatability was determined as intraday precision whereas intermediate precision was determined by carrying out inter-day variation for the determination of swertiamarin at four different concentration levels of 100, 150, 200 and 250 µg/ml in triplicates.

Reproducibility

Reproducibility of the proposed method was checked by analyzing precision on a different instrument, which was analyzed by another person in different laboratory. Both intraday and intermediate precision was calculated at four different concentration levels of 100, 150, 200 and 250 µg/ml in triplicates.

LOD and LOQ

LOD and LOQ were determined by standard deviation (SD) method. LOD and LOQ were determined by injecting blank sample to the chromatograph in triplicates, peak area of this blank was recorded. LOD and LOQ were determined using the slope of the calibration curve and SD of the blank sample using following formulae:

\[
LOD = 3.3 \times SD / S
\]

\[
LOQ = 10 \times SD / S
\]

Where SD is the standard deviation of the blank response and S is the slope of the calibration curve.

Robustness

Robustness of the method was determined by changing the flow rate (1.1 and 0.9 ml/min) of mobile phase. The change in retention time (R_t) was recorded and % RSD was calculated.

Quantification of swertiamarin in methanolic extract and formulations

The test samples were injected and chromatograms were obtained in same conditions as that of standard swertiamarin. The peak area of the peak corresponding to the 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

Method development

The mobile phase composition was optimized to develop a
suitable and accurate HPLC method for quantification of swertiamarin. Mobile phases tried for this purposes were methanol: water (90:10 %), acetonitrile: water (25:75 %), methanol: water (50:50 %), acetonitrile: water (50:50 %) and acetonitrile: water (75:25 %). The chromatogram obtained with methanol: water (90:10 %) solvent system was found to have very good symmetry (1.15), with lowest R_t (10.15 min) and sharp well defined peak (Figure 1). Therefore the mixture of methanol: water (90:10 %) was optimized as the mobile phase. The drug was stable for a period of 48 h storage at laboratory temperature and under refrigerator temperature in methanol: water (90:10 %) mixture.

**Calibration curve**

The calibration curve area versus concentration (µg/ml) was found linear in the range of 50-300 µg/ml. Values of mean area with corresponding concentration, their standard deviation, % RSD and standard error are shown in Table 1. Statistical calculations were done at 5% level of significance. One-way analysis of variance (ANOVA) statistical test was performed to compare these results. The linear regression data for the calibration curve showed a good linear relationship over the concentration ranges of 50-300 µg/ml with respect to peak area as shown in Table 2. The correlation coefficient value (R^2) was highly significant (Table 2) (p<0.05). No significant differences were observed in the slope of standard curves (p>0.05). The retention time and asymmetry factor were found to be 10.15±1.52 min and 1.15 ±0.16 respectively.

**Validation of the Method**

**Linearity**

The linearity range of swertiamarin was obtained as 50-300 µg/ml as shown in Table 1. The regression equation was Y= 25653X+490912 with correlation coefficient (R^2) of 0.998 (Table 2).

**Accuracy as recovery**

The accuracy of the proposed method was calculated by recovery analysis which afforded recovery of 99.16-99.89 % after spiking the additional standard drug solution to the previously analyzed test solution. The values of % recovery, % RSD and

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Mean area ±SD (n=3)</th>
<th>% RSD</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1776453±1956</td>
<td>1.10</td>
<td>1129</td>
</tr>
<tr>
<td>75</td>
<td>2334156±9876</td>
<td>0.42</td>
<td>5702</td>
</tr>
<tr>
<td>100</td>
<td>3067541±16498</td>
<td>0.53</td>
<td>9525</td>
</tr>
<tr>
<td>150</td>
<td>4334512±155999</td>
<td>0.35</td>
<td>8983</td>
</tr>
<tr>
<td>200</td>
<td>5764123±27321</td>
<td>0.47</td>
<td>15774</td>
</tr>
<tr>
<td>250</td>
<td>6934561±48865</td>
<td>0.70</td>
<td>28213</td>
</tr>
<tr>
<td>300</td>
<td>8084151±59876</td>
<td>0.74</td>
<td>34570</td>
</tr>
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</table>

**Table 1:** Calibration curve of swertiamarin in methanol: water (9:1) mixture.

<table>
<thead>
<tr>
<th>Linearity range (µg/ml)</th>
<th>50-300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression equation</td>
<td>Y= 25653X+490912</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.998</td>
</tr>
<tr>
<td>Slope±SD</td>
<td>25653 ± 297.56</td>
</tr>
<tr>
<td>Intercept±SD</td>
<td>490912± 564.25</td>
</tr>
<tr>
<td>Slope without intercept±SD</td>
<td>28022±321.43</td>
</tr>
<tr>
<td>Standard error of slope</td>
<td>171.80</td>
</tr>
<tr>
<td>Standard error of intercept</td>
<td>325.77</td>
</tr>
<tr>
<td>95% confidence interval of slope</td>
<td>26392.25-24913.75</td>
</tr>
<tr>
<td>95% confidence interval of intercept</td>
<td>492313.78-489510.22</td>
</tr>
<tr>
<td>Bias of intercept</td>
<td>-0.0612</td>
</tr>
</tbody>
</table>

**Table 2:** Linear regression data for the calibration curve (n=3).
standard error are shown in Table 3, which indicated the good accuracy of the proposed method.

**Precision**

Precision was considered repeatability (intraday precision) and intermediate precision. Results of repeatability and intermediate precision were expressed in terms of % RSD and are shown in Table 4. The low values of % RSD indicated the repeatability and intermediate precision of the proposed method.

**Reproducibility**

Reproducibility of the proposed method was checked by obtaining precision of the method in another laboratory using different instrument and analyzed by another person but in the same condition. Both intraday and interday precision was examined in labs. There were no significant differences observed in the % RSD values of intraday and interday precision, which indicates the reproducibility of the method Table 5.

**LOD and LOQ**

LOD and LOQ of the proposed method were determined by the standard deviation method and were found to be 17.25 and 56.92 µg/ml respectively, which indicated that the proposed method can be used in wide range for detection and quantification of swertiamarin effectively.

**Robustness of the method**

Robustness was determined to evaluate the influence of small but deliberate variation in the chromatographic conditions for the determination of swertiamarin. There was no significant change in the R<sub>t</sub> of swertiamarin by changing the flow rate (Table 6). Low value of the % RSD indicated the robustness of the method as shown in Table 6.

**Quantification of swertiamarin in extracts of E. littorale**

The peaks of swertiamarin from sample solution were identified by comparing their R<sub>t</sub> obtained from the peaks with those of standard. HPLC profile of the 60 % methanolic extract of the E. littorale was developed through the same condition as estimation of standard swertiamarin (R<sub>t</sub> value 10.15 min). The R<sub>t</sub> value of swertiamarin was 10.17 min in extract as shown in Figure 2. Some other peaks were also obtained in extract at different values of R<sub>t</sub> because of presence of some impurities in the extract as shown in Figure 2. The peak purity of extract was confirmed by superimposing the peaks of standard and extract. It was observed that extract of E. littorale collected from UA zone showed good content of swertiamarin followed by samples from MP and RAJ regions. The swertiamarin was quantified in extract of E. littorale.

<table>
<thead>
<tr>
<th>Excess drug added to analyte (%)</th>
<th>Theoretical content (µg/ml)</th>
<th>Conc. Found (µg/ml) ±SD</th>
<th>% Recovery</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>98.89±1.98</td>
<td>98.89</td>
<td>2.00</td>
</tr>
<tr>
<td>50</td>
<td>150</td>
<td>149.10±2.96</td>
<td>99.40</td>
<td>1.98</td>
</tr>
<tr>
<td>100</td>
<td>200</td>
<td>198.32±3.76</td>
<td>99.16</td>
<td>1.89</td>
</tr>
<tr>
<td>150</td>
<td>250</td>
<td>248.95±4.93</td>
<td>99.58</td>
<td>1.98</td>
</tr>
</tbody>
</table>

Table 3: Accuracy of the proposed method (n=3).

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Repeatability (Intraday precision)</th>
<th>Intermediate precision (Interday)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean area ±SD (n=3)</td>
<td>Standard error</td>
</tr>
<tr>
<td>100</td>
<td>3023543±14123</td>
<td>8154.15</td>
</tr>
<tr>
<td>150</td>
<td>4212653±13876</td>
<td>8011.54</td>
</tr>
<tr>
<td>200</td>
<td>5732876±26987</td>
<td>15581.40</td>
</tr>
<tr>
<td>250</td>
<td>6889765±47876</td>
<td>27642.03</td>
</tr>
</tbody>
</table>

Table 4: Precision of the proposed method.

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Flow rate (ml/min)</th>
<th>Original</th>
<th>Used</th>
<th>Level</th>
<th>Mean area±SD (n=3)</th>
<th>Standard error</th>
<th>% RSD</th>
<th>Mean R&lt;sub&gt;t&lt;/sub&gt; ±SD (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td></td>
<td>0.9</td>
<td>-0.10</td>
<td>2945387±24543</td>
<td>14170.32</td>
<td>0.83</td>
<td>10.45±1.55</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>0</td>
<td>2989654±22387</td>
<td>12925.51</td>
<td>0.74</td>
<td>10.25±1.21</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.1</td>
<td>+0.10</td>
<td>3165873±29821</td>
<td>17217.66</td>
<td>0.94</td>
<td>10.10±1.46</td>
<td></td>
</tr>
</tbody>
</table>

Table 6: Robustness of the method by changing flow rate.
Using regression equation and value was found to be 11.70 %, 9.20 % and 7.10 % w/w in UA, MP and RAJ samples respectively.

Quantification of swertiamarin in marketed formulations

A single HPLC peak was observed at the same R_t in the samples of marketed formulations A and B. There was no interaction between the swertiamarin and other excipients present in the marketed formulations. The swertiamarin content was found to be 7.50 % and 8.11 % w/w in formulation A and B respectively. The value of % RSD was also too low which indicated the suitability of this method for the routine analysis of swertiamarin in marketed formulations.

Applications

The method was used to determine the presence swertiamarin in *E. littorale* from three different parts of the India. The swertiamarin was present in all the three extract and in marketed formulations, but there was considerable variation in the amounts present. It may be because of genetic variability, source of plant as well as external factors like seasonal and environmental variations, drying processes and storage conditions cannot be ruled out.

Conclusions

The proposed validated HPLC method is suitable for the quantification of swertiamarin in both methanolic extract and marketed formulations. Therefore, this method can be successfully used for the routine analysis of swertiamarin in both crude drugs and prepared formulations without any interference which can be explored for standardization and quality control of raw materials and marketed herbal products of traditional system of medicine containing *E. littorale* as an ingredient.

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