

IMPROVED RP-HPLC METHOD FOR QUANTITATIVE ESTIMATION OF STEVIOSIDE IN *STEVIA REBAUDIANA* BERTONI BURM

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ABSTRACT

RP-HPLC method with UV array detection was established for the determination of stevioside, an extract of herbal *S. rebaudiana* plant. The stevioside was separated using isocratic solvent system consisting of methanol and 0.1% orthophosphoric acid (v/v) in water (70:30) at flow rate of 1.0 ml/min and the detection wavelength of 219 nm. The method was validated for linearity, precision, accuracy, limit of detection (LOD), and limit of quantitation (LOQ). The linearity of the proposed method was obtained in the range of 5.0-75 µg/ml with regression coefficient of 0.9999. Intraday and interday precision studies showed the relative standard deviation less than 2.5%. The accuracy of the proposed method was determined by a recovery study conducted at 3 different levels. The average recovery was 97-99%. The LOD and LOQ were 0.02 and 0.05 µg/ml, respectively. The content of stevioside obtained in the dried leaves powder was within the ranges of 6.83 – 7.91% and 1.7 – 2.9 % w/w, respectively. The proposed method is simple, sensitive, yet reproducible. It is therefore suitable for routine analysis of stevioside in *S. rebaudiana* Bertoni.

Keywords: *Stevia rebaudiana*; stevioside; Quantitative HPLC.

1. INTRODUCTION

Stevia rebaudiana Bertoni belonging to family Asteraceae is a rich source of herbal sugar, stevioside and rebaudioside A. A native to South America, the plant is being cultivated in China and Southeast Asia for last few decades signifying the health related benefits of use of plant and materials of natural origin. Stevia leaves contain diterpene glycosides namely, stevioside (Figure 1), rebaudiosides A-F, steviolbioside, and dulcoside A, which are responsible for the typical sweet taste¹. Stevioside is the predominant sweetener, accounting for 3-8% (w/w) of dried leaves with a sweetening power 300 times that of sucrose and is known to be noncaloric². Steviosides can be degraded to its major metabolite, steviol, by intestinal bacterial microflora of human being³⁻⁵. Stevioside has been shown to have therapeutic value as an antihypertensive or antihyperglycemic (which subordinate the blood sugar) agent⁶⁻¹⁰. The available data indicates that stevioside is nontoxic, nonmutagenic and noncarcinogenic in various mammalian species^{11,12}.

High performance liquid chromatography (HPLC), especially reverse-phase chromatography, is one of the most commonly used methods for the determination of stevioside in plant material and food samples. It is also possible to use thin-layer chromatography, near-infrared spectrometry, VIS

spectrometry¹³ and capillary electrophoresis¹⁴ for the determination of the diterpene glycosides. Ahmed and Dobberstein developed an HPLC method for the determination of eight diterpene glycosides¹⁵. Satisfactory resolution was achieved on two protein columns in series, after the plant material was extracted with chloroform prior to methanol extraction. Macapugay *et al.* have reported on HPLC separation of the same compounds, using an NH₂ phase-bonded column with a linear gradient¹⁶. Extraction of *S. rebaudiana* leaves with chloroform and methanol in a Soxhlet apparatus similar to the method described above was used as a pre-separation step. Nikolova-Damyanova *et al.* have presented two methods, normal phase HPLC and silica gel thin-layer chromatography with densitometry, applied for the separation and quantification of Stevioside and rebaudioside A in *S. rebaudiana* leaves. The glycosides were isolated from the plant by a three fold extraction, each time with fresh boiling water. The mobile phase consisted of 1-propanol, ethyl acetate and water. The duration of analysis was the main difference between the two methods used, HPLC and TLC¹⁷. Nishiyama *et al.* have reported on quantitative analysis of stevioside in the leaves of *S. rebaudiana* by near-infrared reflectance spectrometry and by HPLC using C₁₈ column and a mobile phase consisting of methanol and NaOH¹⁸. In this case, the dry plant material was extracted with water close to boiling point by three consecutive infusions for the complete extraction. After the adjustment of pH of the liquid extract to 9, the samples were filtered through a Millipore membrane and injected immediately for chromatography. The derivatization of stevioside has been reported by Ahmed *et al.*^{19, 20}. Here, the steviolbioside and rebaudioside B were reacted with *p*-bromophenacyl bromide and crown reagent to yield the corresponding chromophore esters, which were separated and quantified by HPLC. The separation was carried out using a C₁₈ column using a mixture of acetonitrile and water as the mobile phase. The extraction of *S. rebaudiana* leaves with a mixture of methanol and water and the consecutive extraction with a mixture of water, ethanol, ethyl acetate and cyclohexane has been reported by Gagliardi *et al.* A linear gradient of the mobile phase (methanol-acetonitrile-water), UV detection (217 nm) and a C₁₈ column was used²¹.

Although, a reasonably good literature exists on the separation of stevioside as seen in the foregoing section, however, no reports have been found describing the separation of stevioside using orthophosphoric acid in the mobile phase system under acidic condition.

The aim of this work was to develop an alternative method of analysis, requiring less time and solvent consumption, and using an extraction procedure that is more benign and user friendly. The proposed procedure can be used in the quality control of stevioside content in dried leaves of *S. rebaudiana* before processing, in a selective sampling program; when searching for plants of higher yield in diterpene glycosides content; or when a large number of samples are sent to the laboratory for analysis.

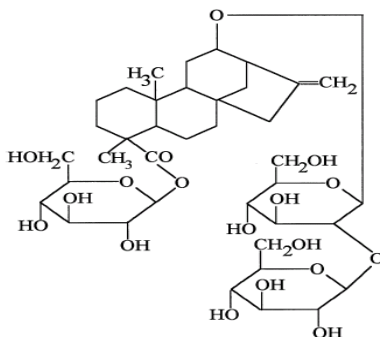


Figure 1. Structure of Stevioside

2. EXPERIMENTAL

2.1 Plant Material and chemicals: Dried leaves of stevia were purchased from two different herbal suppliers Amrut-Lal (A) and All India stores (B) from the local market in Mumbai. The sample was authenticated and the sample specimen was preserved. The dried leaves were ground into powder, passed through a sieve (20 meshes). The samples were separately kept in air tight container and protected from light until used. HPLC grade methanol from Merck Specialty Private Ltd (Mumbai, India) and orthophosphoric acid from s. d. fine-chem limited were used (Mumbai, India). Deionized water was obtained from in-house Milli-Q Nanopure (Millipore, Bedford, MA, USA).

2.2. Method

2.2.1 Preparation of extract: 100 g of dried leaves powder was extracted with 1 liter of water with stirring (120 rpm) at 55°C for 2 hr. The process was repeated for one more time for complete extraction of stevioside. The two fractions were pooled together and concentrated by distillation to get dry residue.

2.2.2 Chromatography: Agilent (Germany) HPLC system, consisting of a model G1329A standard auto-sampler, model G1316A thermostat column, model G1322 A vacuum degasser, quaternary pump, model G1314B variable wavelength detector, was used. The separation was achieved on a stainless steel silica based Zorbax Eclipse XDB-C18 column (ϕ 4.6 mm \times 150 mm, 5 μ m). The absorption was measured at 219 nm for stevioside. The chromatographic data was recorded and processed with EZChrom Elite software.

2.3 Sample preparation

2.3.1 Standard solution preparation: Standard stevioside 10 mg was accurately weighed and transferred to a 10 ml volumetric flask and the volume was made with water. Solutions of 10, 20, 30, 40 and 50 μ g/ml were made by transferring the aliquot from stock solution and the volume was made with water in each case. Further standard solutions were prepared freshly each day by appropriate dilution of stock solution with water for intraday as well as interday analysis.

2.3.2 Test sample preparation: 25 mg of water extract was accurately weighed and transferred to a 25 ml volumetric flask and the volume was made by distilled water. The final stock solution of 100 μ g/ml concentration was made by transferring 1 ml of above solution to 10 ml volumetric flask and the volume was made with distilled water. Then 10 μ l of the stock solution was subjected to HPLC analysis and the concentration of stevioside was calculated based on the calibration curve equation.

2.4 Validation of the Method

Validation of the analytical method was done according to the International Conference on Harmonization guideline (ICH, 1996). The method was validated for linearity, precision, and accuracy, limit of detection (LOD) and limit of quantitation (LOQ).

2.4.1 Linearity: Linearity was determined by using stevioside standard solution. 5 to 75 μ g/ml of the standard solution was prepared ($n = 3$). The calibration graphs were obtained by plotting the peak area versus the concentration of the standard solutions.

2.4.2 Precision: The precision was determined by analyzing 25, 50, and 75 μ g/ml of standard solution of stevioside ($n = 3$) on the same day for intraday precision and on 3 different days for interday precision by the propose method. The precision was expressed as relative standard deviation (RSD).

2.4.3 Accuracy: The accuracy of the method was tested by performing recovery studies at 3 levels of stevioside reference standard added to the samples. Three different volumes (0.5, 1, and 1.5 ml) of the standard solution (containing 200 µg/ml of stevioside in water) were added to the sample solution (150 µg /ml) and analyzed by the proposed HPLC method. The recovery and average recovery were calculated. Three determinations were performed for each concentration level.

Recovery (%) = (amount found - amount contained) / amount added x 100

2.4.4 Limit of Detection (LOD) and Limit of Quantitation (LOQ): According to the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use recommendations, the approach based on SD of the response and the slope were used for determining the detection and quantitation limits.

3. RESULTS AND DISCUSSION

3.1 Chromatography: Under the current conditions, stevioside along with other phytoconstituents of *S. rebaudiana* leaves extract were eluted within 10 min. The peaks in the HPLC chromatogram of leaves extract were identified by comparing the retention time and UV spectra of stevioside in the samples with stevioside standard. The peak purity was above 97 %. Figure 2. shows the chromatograms of stevioside standard and water extract of *S. rebaudiana* sample at 219 nm. The quantification data is as shown in Table I.

3.2 Validation of chromatographic method: The method was validated for its linearity, precision, accuracy, LOD and LOQ. The calibration graph for stevioside was within the concentration range of 5.0-75 µg /ml, with a correlation coefficient (r^2) of 0.9999 (Table 1). The interday and intraday precisions of stevioside are presented in Table 2. The results showed acceptable precision of the method, with RSD values much lower than 2.5%. The recovery at 3 different levels of stevioside was 95.69, 97.33, and 98.89% with an average of 97.30% (Table 3). These values indicate the accuracy of the method. The LOD and LOQ for stevioside were found to be 0.06 and 0.17 µg/ml, respectively, which indicate a high sensitivity of the method which was calculated by using the following formulae.

LOD = $3.3 \sigma / S$ and LOQ = $10 \sigma / S$.

Where σ is the standard deviation of the response and S is the slope of the calibration plot

3.3 Sample analysis: Stevioside content in the samples of *S. rebaudiana* leaves obtained from two different herbal suppliers in mumbai during April 2011 determined by the newly proposed HPLC method are given in Table 4. The contents of stevioside in the dried powder were 7.87 ± 0.08 and $7.50 \pm 0.04\%$ w/w (Table 4). HPLC chromatograms of both extracts showed similar pattern with a major peak of stevioside at retention time of 3.97 min (Figure 2). The identity of the peak of stevioside in the sample chromatograms was confirmed with the standard and the corresponding retention time.

CONCLUSIONS

The need for quality assurance, including confirmation of the label strength and content uniformity has long been recognized even for herbal medicinal products. A high-performance liquid chromatography method has been developed for the detection and quantitation of stevioside of *S. rebaudiana* extract using UV array detection. Analysis of *S. rebaudiana* samples with the proposed method does assure prolong life of column and system due to lower percentage of acid in the

mobile phase and the stevioside can be quantitated successfully, using standard calibration curve. The method was found to be specific and suitable for routine analysis because of its simplicity and reproducibility. The relative standard deviation for the investigated *S. rebaudiana* extracts indicates that the method is precise and reproducible.

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Table 1. Method of validation parameters for the quantification of stevioside by the proposed method

Parameters	Results
Linear range ($\mu\text{g/ml}$)	5.0-75 $\mu\text{g/ml}$
Regression equation	$y = 4.913x$
Correlation coefficient (r^2)	0.9999
LOQ ($\mu\text{g/ml}$)	0.02
LOD ($\mu\text{g/ml}$)	0.05

Where,

x is the concentration of stevioside in $\mu\text{g/ml}$

y is the peak area at 219 nm

Table 2. Precision and Stability of stevioside

Analyte		Precision (RSD, %)				Stability
		Intra-day (n=3)		Inter-day (n=3)		RSD of P _a (%)
		R _t	P _a	R _t	P _a	
Stevioside	25 µg/ml	2.6	0.33	2.64	0.35	0.31
	50 µg/ml	2.67	0.37	2.68	0.38	0.37
	75 µg/ml	2.69	0.39	2.71	0.41	0.40

Table 3. Recovery of stevioside from *S.rebaudiana*

Analyte	Contained (µg/ml)	Added (µg/ml)	Found (µg/ml)	Recovery (%)	Mean (%)	RSD (%)
Stevioside	78.7	100	164.87	95.76	97.30%	2.25
	78.7	100	167.69	95.81		
	78.7	100	165.58	95.49		
	78.7	50	125.62	97.29		
	78.7	50	124.76	96.81		
	78.7	50	125.12	97.89		
	78.7	10	88.67	98.56		
	78.7	10	88.52	99.11		
	78.7	10	88.59	98.92		

Table 4. The content of stevioside in dried powder of *S. rebaudiana* leaves by the proposed method

Sample	Stevioside content (% w/w) in dried leaves powder
Herbal supplier A	7.87 ± 0.08
Herbal supplier B	7.50 ± 0.04

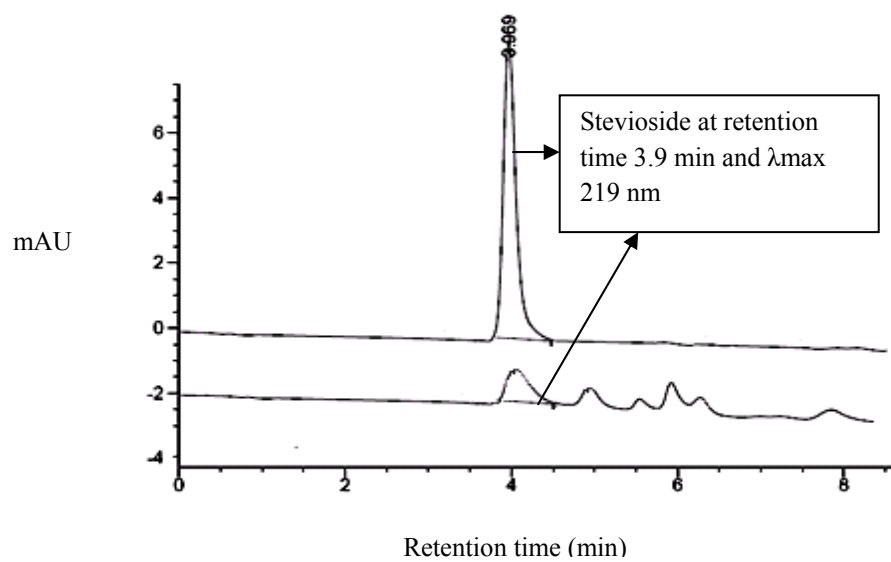


Figure 2. Chromatograms of standard stevioside (upper chromatogram) and water extract (lower chromatogram) of *S. rebaudiana* leaves.