

Development and Validation of an RP-HPLC Method for Determination of Solasodine, a Steroidal Alkaloid

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ABSTRACT

Objective: The aim of the present study was to optimize an RP-HPLC method for determination of solasodine, a steroidal alkaloid with various pharmacologic properties, in herbal medicinal preparations. **Methods:** Various chromatographic parameters including the composition of mobile phase and its organic and pH modifiers, mode of elution and column temperature were investigated using analytical C₁₈ columns as stationary phase. Acetonitrile and methanol were examined in combination with deionized water and different aqueous buffers as mobile phases. **Results:** A mixture of methanol: KH₂PO₄ buffer (pH 2.5) at the ratio of 75:25 %v/v in an isocratic elution mode produced more symmetric, sharper peaks with better retention times and capacity factor than that of acetonitrile: KH₂PO₄ buffer. The optimum column temperature was found at 25°C. The standard calibration curve showed a very good linearity (R²=0.9966) in the range of 1-25 µg/ml with acceptable inter-day and intra-day precisions and accuracy.

In addition, the LOD and the LOQ of the method were determined at 0.2 and 0.7 µg/mL, respectively. **Conclusion:** This developed and validated method is simple and quite rapid and can be used for routine determination of solasodine in samples such as traditional medicinal preparations.

Key words: Solasodine, Steroidal alkaloid, RP-HPLC, Validation.

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INTRODUCTION

Alkaloids as secondary metabolites have been discovered in many plants in the form of glycoside and glycoalkaloid. Steroidal glycoalkaloids and their aglycones like solasodine are found in Solanaceae family and Solanum genus such as potato (*S. tuberosum*), tomato (*S. lycopersicum*) and eggplant (*S. melongena*). These compounds exist more or less in all parts of the plant including roots, stems, leaves, flowers, fruits and even seeds.¹ Solasodine is considered as a high important compound in food industry, toxicology and pharmacology,²⁻³ and synthesis of steroidal drugs like steroidal hormones.^{4,5} This alkaloid has been used in Chinese, Taiwanese and Korean traditional medicine to regulate the immune system and treat allergic reactions.⁶ In addition, various studies have shown that solasodine can be used for cancer treatment.⁷⁻¹²

Several techniques including potentiometry, UV spectroscopy, Thin Layer Chromatography (TLC), Gas Chromatography (GC), High Resolution GC (HRGC), Capillary Electrophoresis (CE) and High Performance Liquid Chromatography (HPLC) have been reported for qualitative and quantitative determination of glycoalkaloids and their aglycones. Although GC and HRGC have been used for determination of these compounds, it should be noted that glycoalkaloids have low volatility and are also unstable in high temperatures applied in GC.¹³ Van Gelder¹⁴ determined and evaluated the composition of the C27-steroidal alkaloid content of *Solanum species* using HRGC. Cherkaoui S *et al.*¹⁵ have utilized non-aqueous CE with UV and mass spectrometry detection for separation and determination of these alkaloids in plant extracts. They showed that the optimization of electrophoretic parameters, including the nature

of the electrolyte and the organic solvent used lead to a reliable separation of solasodine and solanidine. Hunter *et al.* used HPLC to separate several steroidal alkaloids.¹⁶ Philip *et al.* applied this technique to analyze and separate solasodine, solasonine and solamargine. They investigated and optimized many chromatographic parameters in order to have better separation of these alkaloids.¹³ Kittipongpatana *et al.* significantly increased the sensitivity of HPLC analysis of solasodine in hairy root cultures of *S. aviculare* with an isocratic elution mode.¹⁷ Eanes *et al.* set up three isocratic methods for determination of the pairs of steroidal alkaloids of eggplant.¹⁸ They reported that addition of methanol to acetonitrile portion of the mobile phase with acidic buffers at elevated temperature greatly improved the separation of solasodine and solanidine. Desai *et al.*¹⁹ developed and validated a reversed phase HPLC (RP-HPLC) method for estimation of solasodine in aerial parts of *Solanum nigrum*. They found that a mobile phase composed of acetonitrile and ammonium acetate buffer at pH 7.7 was the best eluent for HPLC analysis of the alkaloid.

Solasodine has a steroidal non-polar part and a weak nitrogenous base part of oxa-azaspirodecane (pKa 7.7)²⁰ in its structure (Figure 1); and it is considered as a lipophilic compound with a log P of about 5.²¹ For reversed phase HPLC analysis of solasodine, it is best to have the analyte in its fully non-ionized form by adjusting the pH of the mobile phase. But for a weak basic and lipophilic compound like solasodine, it would be a big challenge to have the best chromatographic conditions for a symmetrical and sharp peak in chromatogram so that the sensitivity of the

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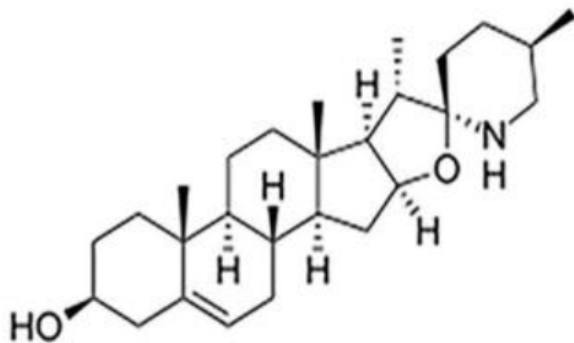


Figure 1: Chemical structure of solasodine.

method is not lost and the column lifetime and the parts of the instrument are not affected and are not damaged as well. As reviewed in the above-mentioned articles, the researchers did not reach a common condition for RP-HPLC analysis of solasodine and the related compounds. Therefore, in the current study we tried to find and to optimize the best chromatographic conditions for RP-HPLC quantitative determination of solasodine, as an important steroidal alkaloid in plants and in herbal medicinal preparations as well.

MATERIALS AND METHODS

Materials

Analytical C_{18} columns were prepared from Phenomenex (USA) and Knauer (Germany) companies. Solasodine standard was purchased from Santa Cruz Biotechnology Inc. (USA); and its 1 mg/ml stock solutions were prepared in HPLC grade methanol. HPLC grade methanol and acetonitrile were purchased from Duksan Pure Chemicals Company Ltd. (South Korea). Deionized water was prepared using a Millipore™ Direct-Q deionizer (USA). NaH_2PO_4 , KH_2PO_4 , H_2PO_4 (NH_4) and Tris-HCl were prepared from Merck Company (Germany). Buffers and mobile phases were filtered using 0.45 μ m-47 mm PTFE filters (Sartorius, Germany) by a Millipore™ vacuum pump filtration set (USA). The pH of aqueous buffers was adjusted with a Metrohm digital pH meter (Switzerland).

HPLC Instrumentation

The analyses were carried out using a Smartline® HPLC system (Knauer, Germany) consisted of Smartline pump 1000, Smartline PDA-UV detector 2600, Smartline manager 5000, Smartline autosamplers 3950, membrane vacuum degasser and column temperature compartment. Separations were performed on C_{18} columns including Knauer® (250×4.6 mm, 5 μ m) (Germany) and Phenomenex® (150×4.6 mm, 3 μ m) (USA). The software ChromGate® version 3.1.7 (Knauer®, Germany) was utilized for data acquisition and processing.

Materials

HPLC method conditions

The HPLC chromatograph used in this study was equipped with a PDA-UV detector; therefore, by UV scanning and evaluating the resulted spectrum during the analysis of solasodine standard solution, the wavelength 205 nm was chosen and set for the UV detector.

Two reversed phase HPLC columns including Phenomenex® C_{18} (150×4.6 mm, 3 μ m) and Knauer® C_{18} (250×4.6 mm, 5 μ m) were examined in this project. By performing several analyses with injection

of 50 μ l of standard solasodine solution using these two cartridges, it was revealed that Knauer® column could produce sharper peaks with asymmetry factor nearer to one; and the analyte band was broadened in chromatograms when using Phenomenex® column. Therefore, Knauer® C_{18} column was chosen for developing, optimizing and setting up the method of solasodine determination. Different temperatures were evaluated and finally 25°C was found the optimum temperature for the column oven.

In different trials, methanol and acetonitrile were examined as organic modifiers in mixture with water or aqueous buffers in the mobile phase. Aqueous solutions of 50 mM NaH_2PO_4 , KH_2PO_4 , $(NH_4)H_2PO_4$ and Tris buffers were used as aqueous portion of the mobile phase. In the final method, methanol: KH_2PO_4 (pH 2.5) (75:25 %v/v) was isocratically considered as the optimum mobile phase.

Preparation of Standard Solution

An amount of 10 mg of solasodine was dissolved in a few ml HPLC grade methanol by sonication for a few min and the volume was made to 10 ml to prepare a stock solution of 1000 μ g/ml. From this solution, various aliquots were taken and diluted with adequate volume of methanol to obtain different concentrations which were used for method development and validation.

HPLC Method Validation

In order to validate the optimized RP-HPLC-UV method, several series of measurements of standard solasodine solutions were carried out and parameters including linear range, precision at intra- and inter-day levels, accuracy, Limit Of detection (LOD) and Limit Of Quantification (LOQ) were calculated according to ICH guidelines.²²

To construct the calibration curve and find out the relevant regression line equation, triplicate analysis of five standard solutions with concentrations of 1, 2, 5, 10 and 25 μ g/ml were injected into the HPLC instrument. The area under solasodine peaks (AUC) were plotted versus the concentrations and the regression line equation was obtained. For intra-day precision, triplicate analyses of fresh-prepared standard solutions at concentrations of 1, 5 and 25 μ g/ml were carried out in one day; and for inter-day level (as intermediate precision), new solutions of the same concentrations were injected into the instrument in three different days. The relative standard deviation (RSD%) of the analyses in one day and during three days were calculated and considered as intra-day and inter-day precisions. To calculate LOD and LOQ, six analytical blanks were analyzed and the signal from noise observed at or close to the retention time (t_r) of the analyte was measured and considered as a criterion. Formulas 1 and 2 were used to calculate LOD and LOQ.

$$(1) \text{ LOD} = 3.3SD_{\text{blank}}/\text{Slope}$$

$$(2) \text{ LOQ} = 10 SD_{\text{blank}}/\text{Slope}$$

SD_{blank} : standard deviation of blank solutions

Slope: the slope of the standard calibration line

RESULTS

Mobile Phase Selection

Mobile phase selection was the first step to optimize the chromatography method in this study. Methanol and acetonitrile were individually tested in combination with 50 mM solutions of different phosphate buffers including NaH_2PO_4 , KH_2PO_4 and $(NH_4)H_2PO_4$ adjusted at pH 2.5 and 50 mM TRIS buffer at pH 7.

First, mixtures of acetonitrile and each buffer were evaluated in three ratios of 70:30, 60:40 and 50:50 %v/v. The solasodine peak was broadened and had high tailing factor in all three tested ratios for mobile phase mixtures of acetonitrile: NaH_2PO_4 , acetonitrile: $(NH_4)H_2PO_4$ and acetonitrile: KH_2PO_4 . In the case of acetonitrile: TRIS buffer, the solasodine

peak was not observed at all during the course of analysis in all three mobile phase ratios. Then, several mobile phases containing methanol and each of the four above-mentioned buffers with similar ratios of 70:30, 60:40 and 50:50 v/v% were examined. Similar to the case of acetonitrile: Tris, the solasodine peak was not observed in any of the methanol: Tris buffer ratios during the considered analysis runtime as well. When using methanol: NaH_2PO_4 mixture at ratio of 70:30 v/v%, solasodine was observed as a wide peak at 15.8 min with a tailing factor much more above one (Figure 2-a). Solasodine peak was not observed in other two ratios of methanol: NaH_2PO_4 at all.

The t_R of the analyte with the eluents of methanol: $(\text{NH}_4)_2\text{H}_2\text{PO}_4$ and methanol: KH_2PO_4 was higher than that of the mixture of acetonitrile with the same buffers, which is relevant to the greater potency of acetonitrile in comparison with methanol in reversed phase chromatography. In contrast, with the replacement of methanol instead of acetonitrile, the shape, width and symmetry of the solasodine peak were considerably improved (Figure 2-b and 2-c).

Methanol: KH_2PO_4 mixtures in all three ratios had more satisfactory results compared to the results of other examined mobile phase mixtures. Methanol: KH_2PO_4 at the volume ratio of 70:30 (pH 2.5) resulted in a

relatively reasonable t_R , good peak symmetry and lower width than the other tested mixtures.

In order to evaluate the effect of reduction of the buffer portion in the mobile phase, volume ratios of 75:25 and 80:20 for methanol: KH_2PO_4 were examined. The solasodine peak was symmetrical and had a suitable width in the case of 75:25 ratio; however, further decrease in the percentage of KH_2PO_4 buffer led to the creation of more tailing in the peak. All in all, it was found that the best mobile phase was composed of methanol and aqueous KH_2PO_4 buffer with the ratio of 75:25 at pH 2.5 (Figure 2-c).

Selecting the Column Temperature

To choose the optimum heat program for the column oven, different temperatures were applied and the results were evaluated regarding the peak shape and the analysis efficiency parameters. Although solasodine peak was observed earlier at elevated temperatures, it had no significant effect on tailing, band-broadening and peak shape at temperatures above 25°C.

Buffer pH Selection

The results of the study showed that the peaks obtained at pH 2.5 have more suitable t_R and symmetric shape compared to the other pHs, which is discussed later.

Validation Results

The constructed calibration curve in the range of 1-25 $\mu\text{g}/\text{ml}$ is shown in Figure 3 and the linearity parameters of the solasodine analysis method are reflected in Table 2. The correlation coefficient was 0.9966 for triplicate analyses of the calibration series which shows high reliability of this validation parameter. The Results of both intra-day and inter-day precisions are cited in Table 3. Repeatability of the method was proved to be good at low, medium and high concentration levels i.e. 1, 5 and 25 $\mu\text{g}/\text{ml}$, respectively. For intermediate (inter-day) precision, the method showed very good precision at levels of 5 and 25 $\mu\text{g}/\text{ml}$ and acceptable result at level of 1 $\mu\text{g}/\text{ml}$. The accuracy results of the method at examined levels of 1, 10 and 25 $\mu\text{g}/\text{ml}$ were 80.9%, 102.5% and 95.6%, respectively (Table 4). Finally, LOD and LOQ values of the method were calculated about 0.2 and 0.7 $\mu\text{g}/\text{ml}$, respectively. Final optimization of RP-HPLC method for determining of solasodine is shown in Table 1.

DISCUSSION

Table 1: Details of the final optimized RP-HPLC method for determination of solasodine.

Variable	Item/Value
Column Dimensions	250×4.6 mm
Stationary Phase	C_{18} , 5 μm
Column Temperature	25 °C
Mobile Phase (A)	Aqueous 50 mM KH_2PO_4
Mobile Phase (B)	Methanol
(A) to (B) Ratio	25:75 (v/v %)
pH of Mobile Phase	2.5
Mode of Elution	Isocratic
Injection Volume	50 μl
Detector	UV at 205 nm
Solasodine t_R	9.1 min
Solasodine Capacity Factor	2.5

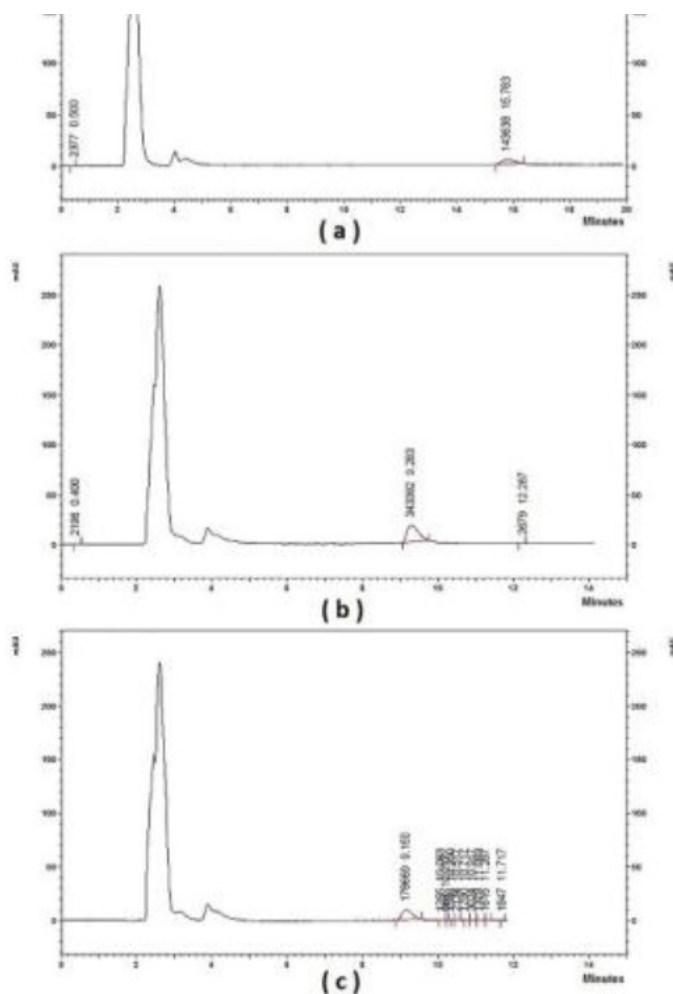


Figure 2: Representative HPLC chromatograms of standard solasodine solutions using different composition of mobile phases at 25 °C. (a) methanol: NaH_2PO_4 (70:30 v/v %), (b) methanol: $(\text{NH}_4)_2\text{H}_2\text{PO}_4$ (75:25 v/v %), (c) methanol: KH_2PO_4 (75:25 v/v %).

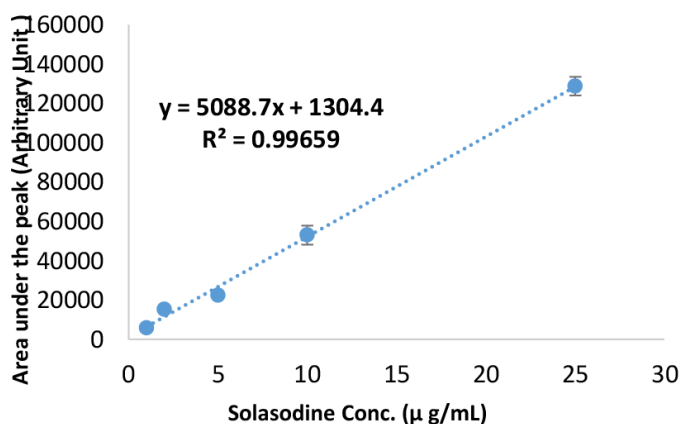


Figure 3: Calibration curve of solasodine in the range of 1 to 25 µg/ml (n= 3, Mean ± SD).

Table 2: Linearity parameters of HPLC method for determination of solasodine.

Parameters	Observations
Linearity range	1-25 (µgml ⁻¹)
Regression equation	Y=5088.7X+1304.4
Correlation coefficient	0.9966
Slope	5088.7
Intercept	1304.4

Table 3: Precision results based on standard deviation percentage at intra-day and inter-day levels.

Conc.(µg/ml)	Precision (RSD %)	
	Intra-day	Inter-day
1	5.7	11.6
5	4.9	3.1
25	3.8	2.3

Table 4: Accuracy results of the RP-HPLC method for solasodine determination.

Expected Concentration (µg.ml ⁻¹)	Measured Concentration (µg.ml ⁻¹)	Accuracy %
1	0.8	80.9
10	10.2	102.5
25	23.9	95.6

Generally in chromatography, parameters such as symmetrical peaks with minimum width, appropriate resolution (Rs), reasonable retention time (t_R) in a minimum run time are desired. These criteria were under consideration while the HPLC method of solasodine determination was being developed in this study.

Solasodine due to its steroidal structure has considerably lipophilic characteristic (log P~5);²¹ hence, a reversed phase chromatography method is suitable for its determination in pharmaceutical and biological samples.²³ As aforementioned, Knauer® C₁₈ column could generate sharper peaks with tailing factor closer to unit for the analyte of interest in comparison with Phenomenex® C₁₈ column. Owing to the fact that

the Knauer® column has a higher level of carbon load and surface coverage and furthermore has a longer length, it generates more theoretical plates than that of the Phenomenex® column, which in turn leads to the creation of sharper peaks with smaller width and better symmetry factor. Meanwhile, there could be more interaction of the secondary amine moiety of solasodine with free silanol groups of the stationary phase in the case of Phenomenex® column (with lower surface coverage and higher free silanol groups in comparison with Knauer® column) which can cause a drastic band-broadening of solasodine peak. Eanes *et al.*¹⁸ used a Nucleosil® C18 column for the same purpose. In our study, the carbon load of Knauer® column was higher than that of Nucleosil® stationary phase²⁴ which makes it more lipophilic, affecting the efficiency of the solasodine analysis.

To have a suitable capacity factor for the analyte of interest while optimizing RP-HPLC method, the analyst has to select an appropriate organic modifier in the mobile phase. Methanol, acetonitrile and tetrahydrofuran (THF) are common organic modifiers as strong eluents in RP-HPLC. THF is restricted to certain conditions because of its corrosive effect on HPLC polymeric plumbing and tubing and due to its higher cost in comparison with the other two solvents. The UV cutoff points of methanol and acetonitrile are 205 and 195 nm, respectively; whereas the lack of extended conjugate double bonds in solasodine gives rise to a poor UV absorption 205 nm for the analyte. These issues bring about a challenge when using methanol and acetonitrile in mobile phase. Despite the λ_{max} of analyte is equal to the cutoff point of methanol, it was observed that solasodine peak had much better efficiency parameters while using this solvent in comparison with that of acetonitrile. This was in agreement with Eanes *et al.*¹⁸ findings, as they used 10% methanol along with acetonitrile and in accordance with Kittipongpatana *et al.*¹⁷ as they used just methanol for organic modifier in the mobile phase. In contrast, Desai *et al.*¹⁹ reported that acetonitrile could give better chromatographic efficiency for solasodine when this solvent was used with 10 mM ammonium acetate buffer at pH 7.7 (65:35 v/v%) to elute the analyte from a C₁₈ column kept at 40°C.

In reversed phase chromatography, it is essential to use aqueous mobile phase in mixture with organic solvents in order to have reasonable retention for the analyte. The addition of water increases the polarization of the mobile phase and decreases the ability to break the lipophilic-lipophilic interaction between the lipophilic analyte and hydrophobic packing material in the column. Meanwhile, as a common rule in reversed phase chromatography, if there are ionizable groups (acidic or basic) in the analyte structure, they should be converted into their non-ionized forms by adjusting the pH of the mobile phase during analysis. This matter brings about a comprehensive interaction between nearly all molecules of analyte and C₁₈ sorbent which prevents the tailing of the chromatography peaks and minimizes band-broadening as well in many cases.²⁵ On the other hand, care has to be taken that the regulatory pH does not damage the silica bonded C₁₈ phase. This kind of phase are usually susceptible to extreme pHs and they tolerate pH range between 2 and 8 (up to 11 are reported for new C₁₈ column generations). Solasodine has a secondary amine group in its structure with a pK_a of 7.7;²⁰ thus, a pH of 9.7 is required to suppress ionization of this basic group up to 99%, which can cause severe damage to the C₁₈ silica bonded phase and reduces the column lifetime dramatically. To overcome this drawback in our research, the pH of the mobile phase was adjusted at 2.5 which is far below the pKa of the compound and makes it ionized instead of being non-ionized while the safety of the column is reassured. At this pH, nearly all molecules of the analyte are in one form and they have their own lipophilic-lipophilic interaction with the C₁₈ phase just through the steroidal rings and the peak tailing is in its lowest amount. In line with our findings in the present study, Kittipongpatana *et al.*¹⁷

showed that pHs close to pKa of solasodine was inappropriate and the efficiency of the analysis was reduced because ionic and non-ionic forms of the analyte exist simultaneously at pHs about the pKa. But Desai *et al.*¹⁹ adjusted the pH of the buffer at 7.7 which is not in agreement with above-mentioned theoretic points and Kittipongpatana *et al.* and our findings in this study as well.

Reversed phase C₁₈ columns usually tolerate temperatures up to 60°C. Increasing the column temperature basically reduces t_R and capacity factor while enhancing the separation performance; but at elevated temperatures the damage to the packing material is high due to the dissolution of the silica bonded stationary phase in the aqueous eluents and the temperature should not be set above ambient temperatures without rational reasoning.²³⁻²⁵ Our findings showed that analyses carried out at 25°C resulted in good efficiency parameters which were quite similar to that of higher temperatures. In contrast, Eanes *et al.*¹⁸ and Desai *et al.*¹⁹ set column oven temperature at 50 and 40°C respectively, which can give rise to harm the stationary phase and to increase the cost in long term.

From the elution mode of view in HPLC method, the use of the gradient elution can basically cause the baseline drift in the chromatograms which in turn can affect the sensitivity and limits of detection and quantification.¹³ Meanwhile, gradient elution programs bring about more maintenance cost for pumps and related devices. Therefore, it must be paid attention not to set this kind of elution in HPLC without any sensible reasoning. In the current work, the chosen mode of elution was isocratic which gave more reliable results during the course of the study.

Regarding the validation parameters, the linearity of the method was successfully proved in the range of 1-25 µg/ml, with an excellent correlation coefficient of 0.9966. The accuracy of the method was determined between 80% and 102% at low and high concentrations which is quite good for a novel method. LOD and LOQ were determined at 0.2 and 0.7 µg/ml, respectively, showing good capability of the method for identification and determination of solasodine as low as fractions of ppm level.

CONCLUSION

Overall, a repeatable, accurate and linear isocratic RP-HPLC method with UV detection at 205 nm was developed for determination of solasodine in herbal medicinal preparations. The analyte retardation was long enough that not to co-elute with early-eluted interfering compounds and was reasonably short enough which could minimize the cost of analysis, especially with using methanol as a low-cost organic solvent in the mobile phase. Therefore, this method could be a good candidate for routine HPLC determination of solasodine in traditional pharmaceutical herbal preparations without any necessity for derivatization of the analyte.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

HPLC: High Performance Liquid chromatography; **RP-HPLC:** Reversed Phase HPLC; **TLC:** Thin Layer Chromatography; **GC:** Gas Chromatography; **HRGC:** High Resolution GC; **CE:** Capillary Electrophoresis; **AUC:** Area Under Curve; **LOD:** Limit Of Detection; **LOQ:** Limit Of

Quantification; **THF:** Tetrahydrofuran; **TRIS:** Tris(hydroxymethyl)aminomethane; **RT:** Retention Time.

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