ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

Vol 7, Suppl 2, 2014

ISSN - 0974-2441

Research Article

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS DETERMINATION OF GALLIC ACID AND BETA SITOSTEROL IN *AMPELOCISSUS LATIFOLIA* (ROXB.) PLANCH

PARAG A. PEDNEKAR[1], VANITA KULKARNI[1], BHANU RAMAN[1]

[1]Department of Chemistry, K.J. Somaiya College of Science and Commerce, Vidyavihar, Mumbai

Email: professor.bhanuraman@gmail.com

Received: 18 March 2014, Revised and Accepted: 19 April 2014

ABSTRACT

Objective: A simple and accurate Reverse phase High Performance Liquid Chromatographic method was developed for simultaneous determination of Gallic acid and Beta sitosterol in methanolic soxhlet leaf extract of *Ampelocissus latifolia* (Roxb.) Planch.

Methods: The separation was achieved using a Surveyor HPLC system (Agilent 1100 series) equipped with U.V. detector. The Column used for Method development and validation was Kromasil 100-5C18 (250x 4.6, 5µm) as stationary phase with a mobile phase comprising of Acetonitrile: Water (95:05) v/v at a flow rate of 1.0 ml min^{-1} and UV detection at 195 nm with a run time of 10.0 min at 30^{0}C.

Results: Regression equations showed good linear relationships (Correlation coefficient r = 0.9969 for Gallic acid & 0.9979 for Beta sitosterol) between the peak area of each marker and concentration. The assay was reproducible with overall intra and inter-day precision. The method has been developed and validated for simultaneous detection of the Gallic acid and Beta sitosterol in methanolic soxhlet leaf extract of *Ampelocissus latifolia*.

Conclusion: The validated method gave good results of system suitability, linearity, LOD & LOQ, precision (Intra-day & Inter-day), accuracy & robustness. The validated HPLC method can be used for a routine quality control analysis.

Keywords: HPLC, Gallic acid, Beta sitosterol, *Ampelocissus latifolia* (Roxb.) Planch.

INTRODUCTION

Quantification of bioactive principles by use of modern analytical tools is essential for establishing the authenticity, creditability, prescription and usage of herbal medicines [1]. *Ampelocissus latifolia* belongs to family Vitaceae i.e. Grape family. It is a large herbaceous climber with tuberous root stock. The stem and branches are hollow and smooth and leaves are circular, broadly heart-shaped with lobes acute and toothed. The plant has antimicrobial and antioxidant potential [2,3].

Gallic acid is a polyphenolic compound having an antioxidant property. Chemically it is a 3,4,5-trihydroxybenzoic acid. Gallic acid and its derivatives are a group of naturally occurring polyphenol antioxidants which have recently been shown to have potential health effects. Gallic acid and its derivatives have neuroprotective effects with free radical scavenging activity [4,5].

Beta sitosterol can stop the absorption of cholesterol in the body and thus reduce the cholesterol levels in the plasma [6,7]. The liver function activity like guanosine diphosphate (GDP) can be improved with beta sitosterol and this can reduce prostate cancer and colon cancer cell growth [8,9]. It can also be the factor used to form the lympho cells and natural killer cells (NK cells) in the immunity process circulation [10]. Beta sitosterol is used in experiments for treating breast cancer and prostate cancer [11].

EXPERIMENTAL

Collection and Authentification of the plant

The leaves of *Ampelocissus latifolia* were collected from Mumbai, Maharashtra. The identification of the plant was done at the Blatter Herbarium, St. Xavier's College, Mumbai. The *Ampelocissus latifolia* (Roxb.) Planch specimen matches with the Blatter Herbarium specimen no. Shah-l of G. L.Shah. The leaves were thoroughly washed with distilled water, dried in an oven at 40°C and grounded into fine powder by using a mechanical grinder.

Chemicals and Standards

HPLC grade Acetonitrile from Qualigens, Mumbai were used. Ultra pure water, generated by use of a Milli-Q System (Millipore), was used for preparation of mobile phase. Standard compounds Gallic acid and Beta sitosterol were from Sigma Aldrich.

Sample preparation

The leaf powder of *Ampelocissus latifolia* (20 gm) was extracted with 250 ml methanol by soxhlet extraction for 8 hours. The extract was concentrated on water bath at 60°C. The dark greenish brown thick liquid obtained was stored in a glass vial in refrigerator.

Selection of Mobile Phase

Depending upon sample solubility, stability and suitability different mobile phase compositions and volume ratio were used for standard and sample solution to get a good resolution and sharp peaks. From the various mobile phases, Acetonitrile: Water (95: 05 %v/v) with U.V. detection at 195 nm was selected.

Diluent Preparation

The Mobile phase was used as diluent.

Standard Stock Solution Preparation

An accurately weighed quantity of Gallic acid and Beta sitosterol was transferred to a volumetric flask, dissolved and diluted to the mark with mobile phase to obtain standard stock solution of 1000µg ml^{-1}.

Apparatus and Chromatographic Parameters

Equipment: Surveyor Agilent 1100 series HPLC system with G1379A Degasser, G1311A Quaternary pump, G1329A Autosampler,

G1330B Thermostat ALS Therm, G1316A Column oven (COLCOM), G1314A U.V. visible detector were used for the experiment.

Column: Kromasil 100-5C18 (250x 4.6, 5 µm)

Flow rate: 1.0 ml min^{-1}

Wavelength: 195 nm

Injection volume: 20 µl

Column oven temperature: 30^{0}C

Run time: 10.0 min

Mode of Operation: Isocratic elution

METHOD VALIDATION

The method was validated according to ICH guidelines for system suitability, linearity, LOD & LOQ, precision (Intra-day & Inter-day), accuracy & robustness [12].

System Suitability

System suitability experiment was performed by injecting consecutive injections (n= 3) of each Gallic acid 50 µg ml^{-1} and Beta sitosterol 100 µg ml^{-1} during the start of the method validation. Values with % RSD < 2 were accepted (Table 1).

Specificity

Specificity is the ability of a method to discriminate between the study analyte(s) and other components in the sample. The resolution between the peaks of the main markers that could be found in extracts of the herb was determined by analysis of chromatograms of the standard solution and the sample solution. It was established by comparing the HPLC retention time and absorption spectra of target peaks from the analysed samples with those of the reference compounds (n=5) (Figure 1).

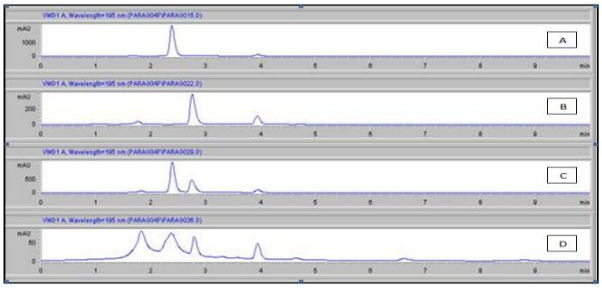

Figure 1. Chromatogram of A: Gallic acid 100 µg ml^{-1}, B: Beta sitosterol 100 µg ml^{-1}, C: Gallic acid 50 µg ml^{-1} and Beta sitosterol 100 µg ml^{-1}, D: methanolic soxhlet leaf extract of *Ampelocissus latifolia*.

Linearity

The linearity between peak area and concentration was analyzed using 2 calibration curves obtained with 2 standard solutions of Gallic acid and Beta sitosterol at 6 different concentrations (n=4) of each standard. The data for peak area v/s concentration were treated by linear regression analysis (Table 2 & Figure 2 & 3).

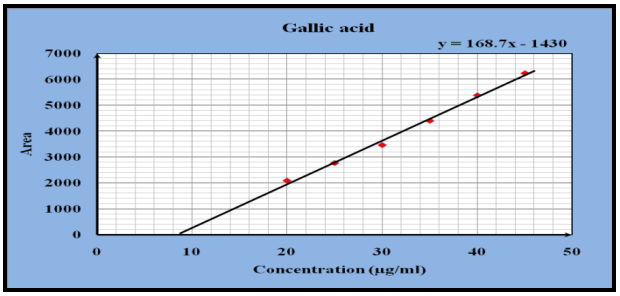

Figure 2. Linearity studies for Gallic acid.

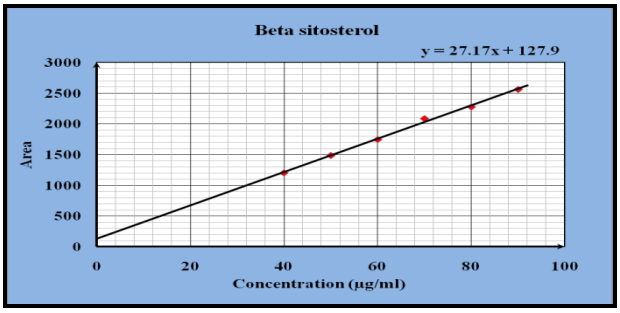

Figure 3. Linearity studies for Beta sitosterol.

Table 1. System Suitability for Gallic acid and Beta sitosterol standards (n= 3).

Compound	Parameter	Acceptance	Average	%RSD
Gallic acid 50 µg ml^{-1}	Retention Time (min)	% RSD < 2	2.38	0.05
	Peak area	% RSD < 2	6607.87	0.66
	Theoretical plates per meter	> 3500	8,972.66	0.43
	Symmetry factor / Tailing factor	< 2	0.76	0.61
	Resolution	>2	3.30	0.34
Beta sitosterol 100 µg ml^{-1}	Retention Time (min)	% RSD < 2	2.74	0.09
	Peak area	% RSD < 2	3067.37	0.81
	Theoretical plates per meter	> 3500	9,206.20	0.33
	Symmetry factor / Tailing factor	< 2	0.62	0.42
	Resolution	>2	3.30	0.34

Table 2. Linear regression data for Calibration curves (n= 4).

Parameters	Gallic acid	Beta sitosterol
Detection wavelength	195 nm	195 nm
Linearity range [µg ml^{-1}]	20-45 µg ml^{-1}	40-90 µg ml^{-1}
Correlation coefficient (r)	0.9969	0.9979
Regression equation	y = 168.71x-1430.7	y = 27.179x + 127.93
LOD	3.726 x 10^{-5} µg ml^{-1}	8.835 x 10^{-3} µg ml^{-1}
LOQ	1.129 x 10^{-4} µg ml^{-1}	0.026 µg ml^{-1}

The equation $y = mx + c$, where y is peak area, m is the slope, x is the concentration, and c is the intercept.

Limit of Detection

Limit of Detection LOD was calculated according to the expression DP x 3.3/IC, where DP is the standard deviation of the response i.e. area of Blank (n=5) and IC is the slope of the calibration curve. By these equations LOD was found to be 3.726 x 10^{-5} µg ml^{-1} for Gallic acid & 8.835 x 10^{-3} µg ml^{-1} for Beta sitosterol.

Limit of Quantitation

Limit of Quantitation LOQ was established by using the expression DP x 10/ IC where DP is the standard deviation of the response i.e. area of Blank (n=5) and IC is the slope of the calibration curve. By these equations LOQ was found to be 1.129 x 10^{-4} µg ml^{-1} for Gallic acid & 0.026 µg ml^{-1} for Beta sitosterol.

Precision

Precision was expressed as the % RSD of the concentrations of each compound. % RSD for Retention time & Peak Area < 2 is accepted.

Repeatability (Intra-day precision)

Injection repeatability was assessed using replicate injections at 80%, 100%, 120% of target concentration of Gallic acid (11.32 µg ml^{-1}) & Beta sitosterol (4.87 µg ml^{-1}) by the proposed method (n=5) (Table 3).

Intermediate precision (Inter-day variation)

The inter-day precision was determined with the sample assayed on 3 different days (n=5) (Table 4-6).

Table 3. Repeatability (Intra-day precision) of Gallic acid & Beta sitosterol (n=5).

Gallic acid	Intra-day			
	Retention time		Peak Area	
Components	**Mean**	**%RSD**	**Mean**	**%RSD**
9.056 µg ml^{-1}	2.38	0.44	542.48	0.68
11.32 µg ml^{-1}	2.35	0.67	1003.86	0.29
13.584 µg ml^{-1}	2.34	0.28	1369.70	0.36
Beta sitosterol	**Intra-day**			
	Retention time		**Peak Area**	
Components	**Mean**	**%RSD**	**Mean**	**%RSD**
3.896 µg ml^{-1}	2.75	0.19	103.16	1.24
4.87 µg ml^{-1}	2.74	0.28	132.34	0.92
5.844 µg ml^{-1}	2.74	0.20	153.2	1.25

Table 4. Intermediate precision (1st Inter-day variation) of Gallic acid & Beta sitosterol (n=5).

Gallic acid	1st Inter-day			
	Retention time		Peak Area	
Components	**Mean**	**%RSD**	**Mean**	**%RSD**
9.056 µg ml^{-1}	2.34	0.28	615.78	0.42
11.32 µg ml^{-1}	2.34	0.20	973.54	0.15
13.584 µg ml^{-1}	2.35	0.21	1304.24	0.20
Beta sitosterol	**1st Inter-day**			
	Retention time		**Peak Area**	
Components	**Mean**	**%RSD**	**Mean**	**%RSD**
3.896 µg ml^{-1}	2.75	0.40	114.12	1.37
4.87 µg ml^{-1}	2.75	0.16	137.24	0.15
5.844 µg ml^{-1}	2.76	0.22	163.42	0.59

Table 5. Intermediate precision (2nd Inter-day variation) of Gallic acid & Beta sitosterol (n=5).

Gallic acid	2nd Inter-day			
	Retention time		Peak Area	
Components	**Mean**	**%RSD**	**Mean**	**%RSD**
9.056 µg ml^{-1}	2.38	0.24	483.98	0.23
11.32 µg ml^{-1}	2.40	1.14	928.98	0.08
13.584 µg ml^{-1}	2.46	1.22	1261.6	0.11
Beta sitosterol	**2nd Inter-day**			
	Retention time		**Peak Area**	
Components	**Mean**	**%RSD**	**Mean**	**%RSD**
3.896 µg ml^{-1}	2.74	0.10	106.88	0.98
4.87 µg ml^{-1}	2.79	1.31	129.08	0.59
5.844 µg ml^{-1}	2.87	1.24	165.54	0.30

Table 6. Intermediate precision (3rd Inter-day variation) of Gallic acid & Beta sitosterol (n=5).

Gallic acid	3rd Inter-day			
	Retention time		Peak Area	
Components	**Mean**	**%RSD**	**Mean**	**%RSD**
9.056 µg ml^{-1}	2.37	0.63	443.9	0.20
11.32 µg ml^{-1}	2.36	1.13	886.28	0.13
13.584 µg ml^{-1}	2.38	1.88	1303.84	1.31
Beta sitosterol	**3rd Inter-day**			
	Retention time		**Peak Area**	
Components	**Mean**	**%RSD**	**Mean**	**%RSD**
3.896 µg ml^{-1}	2.74	0.40	116.44	0.46
4.87 µg ml^{-1}	2.76	1.11	132.52	0.54
5.844 µg ml^{-1}	2.79	1.74	176.48	1.95

Table 7. Accuracy study (n=5).

Standard	Level	Preanalysed sample (µg ml^{-1})	Amount of std added to preanalysed sample (µg ml^{-1})	Total amount Recovered/ Found in sample (µg ml^{-1})	SD	% RSD	% Recovery
Gallic acid	0%	11.32	0	0	0	0	0
	50%	11.32	5.66	16.68	0.07	0.40	94.69
	80%	11.32	9.06	20.80	0.15	0.73	104.73
	100%	11.32	11.32	21.00	0.03	0.16	85.53
Beta sitosterol	0%	4.87	0	0	0	0	0
	50%	4.87	2.44	7.33	0.04	0.60	100.86
	80%	4.87	3.90	9.22	0.05	0.55	111.56
	100%	4.87	4.87	9.32	0.05	0.49	91.30

Table 8. Robustness study (n= 4).

Gallic acid & Beta sitosterol Standard				
Changing Parameter	**Actual Parameter**	**Modification**	**% RSD of Peak areas**	
			Gallic acid	**Beta sitosterol**
Flow rate	1 ml/min	0.8 ml/min	0.21	0.61
		1.2 ml/min	1.34	0.20
Wavelength	195 nm	192 nm	0.27	0.53
		198 nm	1.12	0.97
Temperature	30^{0}C	25^{0}C	0.74	0.55
		35^{0}C	0.33	0.51
***Ampelocissus latifolia* methanolic extract**				
Flow rate	1 ml/min	0.8 ml/min	0.12	0.65
		1.2 ml/min	0.68	0.83
Wavelength	195 nm	192 nm	0.74	0.26
		198 nm	1.44	1.28
Temperature	30^{0}C	25^{0}C	1.72	0.72
		35^{0}C	1.00	0.39

Accuracy

The accuracy was evaluated by means of recovery assays carried out by adding known amounts of the standard to the sample at 3 different levels (50%, 80%, 100%) of the initial concentration of the sample. Each sample was injected 5 times (n=5) (Table 7). Average recoveries were calculated by the formula:

Recovery (%) = {(amount found – original amount)/amount spiked} x 100.

Robustness

The robustness study was carried out to evaluate the influence of small but deliberate variation in the chromatographic conditions for Gallic acid & Beta sitosterol standards and *Ampelocissus latifolia* methanolic extract (n= 4). Average value of %RSD < 2 was acceptable (Table 8).

CONCLUSION

A new HPLC method to simultaneously analyze Gallic acid and Beta sitosterol has been developed and validated for system suitability, linearity, LOD & LOQ, precision (Intra-day & Inter-day), accuracy & robustness. This HPLC procedure provided excellent identification and quantification of these 2 marker compounds present in methanolic soxhlet leaf extract of *Ampelocissus latifolia* with a short analysis time of 10 mins i.e. 2.38 mins for Gallic acid and 2.74 mins for Beta sitosterol. Since both the marker compounds have been the interest of health benefits, the present analytical study could be a potential application to identify and quantify the both the compounds in other plant extracts.

REFERENCES

1. Singh DP, Srivastava SK, Govindarajan R, Rawat AKS. High Performance Liquid Chromatographic determination of Bergenin in different *Bergenia* species. Acta Chromatographica 2007; 19.
2. Pednekar PA, Raman B. Antimicrobial and antioxidant potential with FTIR analysis of *Ampelocissus latifolia* (roxb.) planch. leaves. Asian J Pharm Clin Res 2013; 6(1): 157-162.
3. Pednekar PA, Raman B. Estimation of variation in the elemental contents of methanolic soxhlet leaf extract of *Ampelocissus latifolia* (roxb.) planch. by ICP-AES technique. Int J Pharm Pharm Sci 2013; 5(3): 938-941.
4. Zhongbing L, Guangjun B, Peter S, Belton C, Huiru D. Structure activity relationship analysis of antioxidant ability and neuroprotective effect of gallic acid derivatives. Neurochemistry International 2006; 48: 263–274.
5. Kubo I, Masuoka N, Joung T, *et al*. Multifunctional antioxidant activities of alkyl gallates. The Open Bioactive Compounds Journal 2010; 3: 1-11.
6. Tatu A, Miettinen A, Helena G. Ineffective decrease of serum cholesterol by simvastatin in a subgroup of hypercholesterolemic coronary patients. Atherosclerosis 2002; 164: 147-52.
7. MacLatchy DL, Van Der Kraak GJ. The phytoestrogen Beta sitosterol alters the reproductive endocrine status of goldfish. Toxicol Appl. Pharmacol 1995; 134: 305-12.
8. Zak A, Vecka M, Tvrzicka E, Hruby M, Novak F, Papezova H, *et al*. Composition of Plasma Fatty Acids and Non-Cholesterol Sterols in Anorexia Nervosa. Physiol. Res 2005; 54: 443-51.
9. Awad AB, Fink CS. Phytosterols as anticancer dietary components: Evidence and mechanism of action. J. Nutr 2000; 130: 2127-30.
10. Bouic PJD, Etsebeth S, Liebenberg RW, Albrecht CF, Pegel K, Van Jaarsveld PP. Beta-sitosterol and Beta-sitosterol glucoside stimulate human peripheral blood lymphocyte proliferation: Implications for their use as an immunomodulatory vitamin combination. Int. J. Immunopharmac 1996; 18: 693-700.
11. Awad AB, Chan KC, Downie AC, Fink CS. Peanuts as a source of Beta-sitosterol, a sterol with anticancer properties. Nutr. Cancer 2000; 36: 238-41.
12. ICH Harmonised Tripartite Guideline, Q2 (R1): Validation of Analytical Procedures: Text and Methodology Current Step 4 version, 2005.