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HPLC method validation for simultaneous estimation of madecassoside, asiaticoside and asiatic acid in *Centella asiatica*

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ABSTRACT

A simple, accurate, precise, faster reverse phase HPLC method has been developed and validated for simultaneous estimation of madecassoside, asiaticoside and asiatic acid from extract and raw material of the herb Centella asiatica (L.) Urban (Apiaceae). The linear range for madecassoside, asiaticoside and asiatic acid was 1026-32.06mcg/ml, 1016-31.75mcg/ml and 1024-32.32mcg/ml respectively. The retention time of madecassoside, asiaticoside and asiatic acid was 10.836, 11.9036 and 18.4002 min respectively and the recoveries of three analyte are 107.84%, 113.06% and 104.03% respectively. In the current study the separation of three compounds has been completed within a period of nineteen minute which save the precious time and as well as solvent consumption. The Proposed method can be used for the quality control of the raw material, extracts and assay of these three markers in Centella.

Key words: Centella asiatica, Madecassoside, Asiaticoside, Asiatic acid and HPLC.

INTRODUCTION

In India most of the traditional knowledge about medicinal plants was in the form of oral knowledge that has been eroded or distorted due to the persistent invasions and cultural adaptations[1]. There is a drastic reduction in Monographs on crude drugs and plant products in the Indian Pharmacopoeia 1955 to 1996; one of the reasons is not doing the estimation and validation of the adopted processes. Now-a-days the importance is not only based on the isolation of a compound and its assay but also on the validation of the method which play an important role by the analyst in quality control or quality assurance of the product. The

validation of an analytical procedure is the process of confirming that the analytical procedure employed for a test of pharmaceutics is suitable for its intended use. In other word, the validation of an analytical procedure requires us to demonstrate scientifically that risks in decision by testing caused by errors from analytical steps are acceptably small[2]. HPLC is a modern technique and a chemical standardize technique which is much more reliable and reproducible method for the standardization of both single and compound herbal formulation. It is the most often involved method for estimation of plant constituents. In some cases the original methods which adopted are time consuming, costly, imprecise, and inaccurate more over cumbersome. Hence search is required for a better method where the method should be simple, cheap less time consuming and can be use widely. In this communication the present work tries to demonstrate a new HPLC method validation for simultaneous estimation of madecassoside, asiaticoside and asiatic acid from extract and raw material of *Centella asiatica.*

The drug consists of the dried aerial parts, preferably leaves of *Centella asiatica* (Linn.) Urban (Syn. *C. coriacea* Nannf., *Hydrocotyle asiatica* Linn., *H. lunata* Linn., *H. wighitiana* wild); Fam. Apiaceae. Major bioactive constituents of the plant are triterpenoid saponins *viz*., madecassoside and asiaticoside, and their aglycones *viz*., asiatic acid and madecassic acid [3]. It is commonly used to cure skin diseases, leprosy, diarrhea, amenorrhea, leucorrhea fever and also as a nerve and brain tonic. Recently, antifilarial, cytotoxicity, antitumour and antiulcer activity of this plant has also been recorded [4].

An extensive survey of literature reveals that analysis of madecassic, asiatic acid, medecassoside and asiaticoside has been done by HPLC where the retention time for all these constituents ranges between 15-24 min[3]. Quantitative determination of triterpenes in Centella extract and also in commercial products has been done by HPLC method in which separation and detection occurred after 50 minute[5]. Despite of the minor structural difference between asiaticoside and the madecassoside the saponins were separated successfully by coupling high-speed counter-current chromatography to thin layer chromatography[6]. Quantification of madecassoside, asiaticoside, madecassic acid and asiatic acid in *Centella asiatica* has been done by HPLC-UV[7]. In this we report a new simple, rapid and reliable reverse phase HPLC method for simultaneous estimation of madecassoside, asiaticoside and asiatic acid in the raw material and as well as in the extract which consuming less time and less solvent.

EXPERIMENTAL SECTION

Acetonitrile HPLC grade and Methanol was procured from Qualigens. Orthophosphoric acid AR grade were procured from Rankem. Water ultra pure 18 MOhm resistance HPLC grade was obtained from a sartorious water purification system. Reference standards of madecassoside, asiaticoside and asiatic acid were procured from (R&D) centre NRPL, Bangalore.

Apparatus and Chromatographic condition

Chromatographic condition

HPLC-LC2010A HT, MODEL LC- 2010A, Spin lab ID: S0001/3624 HPLC-LC2010A, MODEL LC- 2010A,Spin lab ID: S00012693 with photo diode array detector M/S. spinco Biotech Pvt.Ltd. A Merck.k GaA, Germany HIbar®RT250-4.6 Prepacked column; Lichrospher®100; RP-18 e (5µm); was used for the separation, a mixture of Acetonitrile and buffer (orthophosphoric acid in 1000ml of HPLC grade water) was use as mobile phase and detection was carried out at 210nm.The mobile phase was filtered through a 0.45 micron membrane filter and degassed. The injection volume was 20 µl and flow rate was maintained at

1.8ml/min. Run time for Standard and sample was 45min. Data acquisition was done using software CFR-21 Part 11 Compliant.

Preparation of standard solutions

The standard mix dilution was prepared by weighing accurately 10.37 mg of madecassoside, 10.27 mg of asiaticoside and 10.35 mg of asiatic acid in the 10ml volumetric flask and make up the volume 10 ml by using methanol i.e. dilution I, II, III, IV, V and VI were made consecutively by dissolving 5ml to 10ml. 1000 µg/ml of concentration of madecassoside, asiaticoside and asiatic acid were prepared by weighing the calculated quantity of reference standard and by dissolving it in sufficient quantity of methanol in a standard flask.

Preparation of sample solutions

Extract and raw material: 100 mg to 1 gm of samples were weighed from Batch no.CA/05006 and CA/05009 and dissolved in methanol with 100ml volumetric flask for extract. For raw material about 3.5 grams to 4 grams of samples were weighed and transferred to a 250 ml of beaker. The solution was extracted with 50ml of methanol by warming on water bath for about 20 min and then transfers the extract to a 250 ml beaker. The procedure was repeated for 4-5 times till the raw material is completely extracted or till the extract is colorless. All the content was calculated using following formula.

$$\% \text{ Purity} = \frac{\text{Sample area X Std wt X Sample dilution}}{\text{Std area X Std dilution X Sample wt}} \text{ X Purity of analyte}$$

RESULTS AND DISCUSSION

Validation of Linearity

Under the experimental conditions described, linear calibration curves of madecassoside, asiaticoside and asiatic acid were obtained throughout the concentration range. Linearity is determined by injecting series of standard at about 6 different concentrations of 5 replicates that span between 50-200% of the expected working range.

The linear range for madecassoside, asiaticoside and asiatic acid was (1026-32.06mcg/ml), (1016-31.75mcg/ml) and (1024-32.32mcg/ml) respectively. Coefficients of correlation were less than 0.99 r^2.

Validation of Specificity

The specificity of the analyte was checked by peak purity or by the resolution with a concentration range of 100-300mcg/ml for individual standards. When mixed with the other related compounds. The relevant chromatogram spectra were recorded to show the specificity. The Retention time, resolutions were obtain from chromatogram and RRT was calculated. Resolution > 2% was obtained, the developed RP-HPLC method was found to be specific. The retention time graphically represented in graph 1 and graph 2 represented the peak for blank sample. Relative retention time and resolution are tabulated in table 1 & 2.

Table 1: Individual standard solution

Parameters	Retention time
Madecassoside	10.836
Asiaticoside	11.9036
Asiatic acid	18.4002

Table 2: Standard mix dilution

Replicate	Madecassoside	Asiaticoside	Asiatic acid
1	282942	292015	656197
2	283274	290308	652411
3	283265	291120	657613
4	283111	291808	657458
5	283180	291375	656847
Average	283154.4	291325.2	656105.2
SD	136.1885	668.6185	2139.471
RSD	0.048097	0.229509	0.326087

Validation of Precision

The precision of the method was demonstrated by repeatability for system, sample solution, extract raw material and standard solution. Reproducibility studies by assaying the sample and comparing the percentage purity of the replicates by same as well as different analyst. Response of the drug peaks, and percentage relative standard deviation were calculated and presented in Table 3, 4, 5 and 6.

Table 3: Peak area of madecassoside, asiaticoside and asiatic acid

Parameters	Analyte		
	Madecassoside	Asiaticoside	Asiatic acid
Retention time	10.7686	11.6372	18.527
RRT	1.0	1.0806	1.72
Resolution	0.0	5.376	46.810

Table 4: Retention time of Madecassoside, Asiaticoside and Asiatic acid

Replicate	Madecassoside	Asiaticoside	Asiatic acid
1	10.646	11.515	18.406
2	10.633	11.502	18.402
3	10.653	11.521	18.414
4	10.669	11.536	18.42
5	10.778	11.638	18.478
Average	10.6758	11.5424	18.424
SD	0.058589	0.054821	0.030984
RSD	0.548804	0.47495	0.168171

Table 5: Relative standard deviation of samples analyzed by the same analyst

Batch No	Analyte	% of Replicate-1	% of Replicate-2	RSD
CA/05009-10%(500mg)	Madecassoside	10.1816	10.2127	0.2156
	Asiaticoside	11.2051	11.1414	0.4031
	Asiatic acid	2.7996	2.7776	0.5578
CA/05009-10%(100mg)	Madecassoside	10.5468	10.5468	0.6418
	Asiaticoside	11.7512	11.7512	0.1922
	Asiatic acid	2.7985	2.7985	0.2024
CA/05009-10%(200mg)	Madecassoside	10.202	10.191	0.0768
	Asiaticoside	11.437	11.3915	0.2818
	Asiatic acid	2.7813	2.7838	0.0635
CA/05009-10%(400mg)	Madecassoside	10.035	10.2386	1.4202
	Asiaticoside	11.1339	11.1521	0.1154
	Asiatic acid	2.743	2.767	0.6088
	Madecassoside	11.3396	11.3235	0.1004

CA/05009-10%(600mg)	Asiaticoside	12.784	12.784	0
	Asiatic acid	3.12523	3.1247	0.01199
CA/05009-10%(800mg)	Madecassoside	11.1067	11.1851	0.4973
	Asiaticoside	12.1187	12.2019	0.4837
	Asiatic acid	2.9954	3.0072	0.2778
CA/05006-1%(1000mg)	Madecassoside	1.4406	1.4437	0.1519
	Asiaticoside	1.1223	1.2248	0.104
	Asiatic acid	0.5637	0.5635	0.025
QC Ref.no/06050 0039	Madecassoside	0.5978	0.5986	0.0946
	Asiaticoside	0.4646	0.4632	0.2134
	Asiatic acid	0.1397	0.1378	0.9682
QC Ref.no/06060 00187	Madecassoside	0.3744	0.3707	0.7021
	Asiaticoside	0.3114	0.3056	1.329
	Asiatic acid	0.223	0.2221	0.2859

Table 6: Relative standard deviation of samples analyzed by different analyst

Batch No	Reported value	Obtained value	RSD
CA/05009-400mg	11.1	11.1339	0.2156
CA/05009-200mg	11.1	11.4144	1.974
CA/05009-500mg	11.1	11.1719	0.4565

Validation of accuracy

Accuracy of an analytical method was determined by adding known amount of analyte to the raw Material, extract and calculated the spike recovery. The concentration ranges of sample solution was prepared which was approximately spaced and span 50 % (lowest concentration) to 200 % (highest concentration) of the expected operating range. Sample was analyzed according to the method and the assay value, % recoveries were reported.

Assay > 10%, < 95; recovery should be 95 to 110 %, Assay > 0.5%, < 7.5; recovery should be 85 to 120 %. The data is tabulated in table number 7.

Table 7: Recovery studies for madecassoside, asiaticoside and asiatic acid

	Madecassoside	Asiaticoside	Asiatic acid
Amount found	1.196mg	1.34 mg	0.352mg
Amount added	1.026 mg	1.016 mg	1.024 mg
Total amount	13.826mg	13.826mg	13.824 mg
Observed value	17.333%	19.28%	10.3%
Theoretical value	16.07%	17.05%	9.95%
% recovery	107.84%	113.06%	104.03%

Table 8: Relative standard deviation

	Madecassoside		**Asiaticoside**		**Asiatic acid**
1	2106.74	1	2175.072	1	4926.015
2	2171.6	2	2229.066	2	5011.7813
3	2207.83	Avg	2293.89	3	5131.6172
Avg	2162.057	SD	2232.676	Avg	5023.137833
SD	51.21624	RSD	59.4912	SD	103.2704909
RSD	2.36		2.66	RSD	2.05

Validation of range

The specific range was derived from the linearity studies and it was calculated from the linearity graph from lower to higher concentration between which the response is linear, accurate and precise. Acceptance criteria were relative standard deviation < 2.5. Range between which the studies are linear is tabulated in table 8 & 9.

Table 9: Concentration range

Reference standard	Concentration range
Madecassoside	513 to 128.625 mcg/ml
Asiaticoside	508 to 127 mcg/ml
Asiatic acid	512 to 128 mcg/ml

Validation of ruggedness

The Ruggedness of an analytical method was determined by analysis of sample by different operating condition and environment with in specified parameters of the assay. The degree of reproducibility of test was then determined as a function of the assay variables. This reproducibility may be compared to the precision of the under normal conditions. 152.6mg of reference standard (CEA/05LOT5) was weighed and diluted with methanol in the 50ml volumetric flask. The sample solution was prepared by weighing the sample 304.8mg (Batch No.CA/05009). The weighed amount was dissolved in methanol in 100ml volumetric flask. The solution was sonicated and finally made the volume 100ml, then it was filtered and used. Acceptance criteria: relative standard deviation<5. The ruggedness for respective batch is tabulated in table 10.

Table 10:Ruggedness for respective batch

Batch no.	%Purity obtain in ruggedness	%Purity obtain in precision	RSD
CA/05009	23.14	24.12	2.93

CONCLUSION

The proposed RP-HPLC method provides for quantitative estimation of asiaticoside and total triterpenes (madecassoside, asiaticoside and asiatic acid) in raw material and extracts of *Centella asiatica* is accurate, precise, linear, rugged, specific, simple, rapid and within the range. In the current study the separation of all the three compounds has been completed within the period of total nineteen minute which save the consuming of time and as well as the solvent. Hence the present RP-HPLC method is suitable for the quality control of the raw materials, extracts and assay of the three Markers in *Centella asiatica*.

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