



BIOANALYTICAL HPLC METHOD DEVELOPMENT AND VALIDATION FOR QUANTIFICATION OF ASIATIC ACID FROM *CENTELLA ASIATICA* LINN.

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ABSTRACT

Centella asiatica Linn. (Family Apiaceae) is widely used in Indian system of medicine. Early there *in vivo* study had developed but oral administration of extract in rat was not developed. The objective of present study is to develop and validation of the bioanalytical method using a HPLC of Asiatic acid. A HPLC system with UV-vis. detector for the quantitative determination of total concentration of Asiatic acid albino wistar rat whole blood. Asiatic acid was extracted with n-hexane:dichloromethane:2-propanol (20:10:1,v/v/v) from whole blood. Chromatographic separation was used RP-C₁₈ column with solvent system consisting of water-methanol in gradient mode and detection at 205nm using PDA detector. For validation of method by standardizing the parameters like LOD, LOQ, sensitivity, selectivity, accuracy and precision, extraction recovery and stability were validated. A calibration curve ranging from 0.25-50µg/ml was shown to be linear and the lower limit of quantification was 0.25µg/ml. The intra-day and inter-day precisions which were determined by five different concentrations ranged from 4.25%-1.06% and 4.03%-1.50% respectively. Extraction recoveries were not less than 73.72%. Whole blood samples containing Asiatic acid were stable for 14 days at -20°C. The method successfully applied to a Pharmacokinetic study in rat after oral administration of *Centella asiatica* extract for three different doses liked 600, 800 and 1000mg/kg. The parameters of Pharmacokinetic obtained were T_{1/2}: 12.1, 12.65, 12.59hrs; T_{max}: 4hrs; C_{max}: 1.8, 2.35, 2.9µg/ml; AUC_{0-t} and AUC_{0-∞}: 17.2 and 23.4µg/ml, 3.23 and 24.82µg/ml, 29.13 and 30.79µg/ml respectively. The developed method was simple, reproducible, sensitive and specific for determination of Asiatic acid from whole blood of rat.

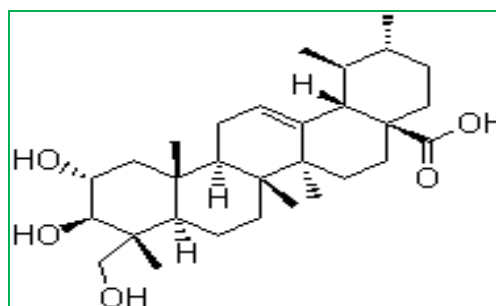
Keywords: Asiatic acid, *Centella asiatica*, HPLC, Pharmacokinetics, Pentacyclic triterpenes.

INTRODUCTION

Centella asiatica Linn. (Family Apiaceae) commonly known as Gotu kola, is widely used in Indian system of medicine from the ancient time. The medical plants are widely used by the traditional medical practitioners for curing various diseases in their day to day by practice.¹ Traditionally *Centella asiatica* is used in malarial fevers, gastric disorders and in hepatic infections. The leaves of the plant have been used as an expectorant, memory enhancer, diaphoretic, antioxidant, anti-ulcer, anthelmintic, antiseptic, analgesic and tonic rejuvenator.² *Centella asiatica* leaves are also used in treatment of breast cancer.³ *Centella asiatica* leaf extract of methanol and ethanol use for the treatment of anti-diabetic.⁴ *Centella asiatica* ethanolic leaf extract were used for the treatment of anti ulcer activity.⁵ Clinical trials have shown that extracts of *C. asiatica* heal wounds, burns and ulcerous abnormalities of the skin.⁶ *Centella* has also been used traditionally as an anti inflammatory and particularly for eczema.⁷

Asiatic acid is a pentacyclic triterpenoid compound found in large quantity of vegetarian fruits and medicinal herbs, presenting several important biological activities. These include anti-microbial, anti-fungal, antioxidant property.⁸ ⁹ Ethanolic extract of *Centella asiatica* has more activity against anti-microbial and anti-fungal which is compare with ciprofloxacin antibiotics as a standard.¹⁰ Other relevant activities such as anti-hepatofibric,¹¹

antinociceptive and anti inflammatory¹² properties are attributed to the presence of Asiatic acid in many plants.



(1S,2R,4aS,6aR,6aS,6bR,8aR,9S,10S,11R,12aS,14bR)-10,11-Dihydroxy-9-(hydroxymethyl)-1,2,6a,6b,9,12a-hexamethyl-2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid)

Figure 1: Chemical structure of Asiatic acid

HPTLC method has been used for the quantification of the Asiatic acid in *Centella asiatica* Linn.¹³ also HPLC method has been studied for the analysis of bioactive triterpenes.¹⁴⁻¹⁵ HPTLC and HPLC method had been developed but not fully validated. Thus a rapid and validated method based on HPLC has been developed for quantitative determination of the compound Asiatic acid in the whole blood of plant extract of *Centella asiatica* Linn. HPLC method had development and their quantitative determination of Asiatic acid form *in vivo* effect in rats at particular time interval after oral administration of drug's different doses.

MATERIALS AND METHODS

Chemicals and Reagents

The herbal extract was prepared by Pharmanza Herbal Pvt. Ltd.; standards were from Chromadex. All dilutions were performed in standard volumetric flasks.

Table 1: List of chemical reagent

Reagent	Purity /Grade	Supplier (Location)
Methanol	HPLC Grade	Rankem, New Delhi
Ethylene dichloride	HPLC Grade	Rankem, New Delhi
n-Hexane	HPLC Grade	E. Merck Ltd., Mumbai, India
Dichloromethane	AR Grade	E. Merck Ltd., Mumbai, India
Water	Double distilled	Pharmanza (INDIA)
Asiatic acid	98.00%	Chromadex (USA)
2-propanol	HPLC Grade	Rankem, New Delhi
P- Toluidine	HPLC Grade	Lobachem, Mumbai

Instrumentation and chromatographic conditions

Chromatographic separation was performed on Shimadzu High Performance Liquid Chromatography equipped with IC-20AT liquid chromatographic pump equipped with HPLC binary gradient with Auto sampler and photodiode detector.

A Phenomenex C₁₈ column (250 × 4.6mm, 5µm) was used for the analysis. The mobile phase comprising a mixture of water and methanol gradient time interval in min liked 0, 0-8, 8-12, 12-18, 18-20, 20-25 and %B 85, 85, 100, 100, 85, 85 respectively was delivered at a flow rate of 1.0 ml·min⁻¹.

Preparation of standard stock solutions

The standard solution of Asiatic acid (1000µg·ml⁻¹) was prepared by dissolving 10mg Asiatic acid (98%) and diluting to 10ml with methanol in a standard volumetric flask. Further serial dilutions were prepared in concentrations of 500, 250, 100 & 10µg/ml. Used of these dilution and prepared whole blood calibration standards concentration while injected all in same biological matrix.

Preparation of sample solution

1ml of whole blood was taken in tubes with drug. To it 250µl of 10M HC was added. This mixture was vortexing for 1min. Then it was put in water bath at 85°C for 2hrs Then extracted with mixture of n-hexane:dichloromethane:2-propanol (20:10:1) ml. The mixture was 6 ml added for extraction than after do vortex for 1min. and this vortex was centrifuge for a 10min. After centrifugation 3.5ml supernatant was transferred to pear shape flask and evaporated to dryness in a water bath at 40°C under a stream of nitrogen. In this residue added 350µl ethylene dichloride (2.5mg/ml in dichloromethane) and 150µl p-Toluidine (2.5mg/ml in dichloromethane) then vortexing for 1min. and put at 30°C for 3hrs after this evaporated to dryness in a water bath at 40°C under a stream of nitrogen. The residue was reconstituted in 0.4ml methanol: water (85:15) with

vortexing for 1min. This solution was injected into the chromatographic system.

Method validation for asiatic acid¹⁶⁻¹⁸

Specificity or selectivity

If the method was intended to quantify more than one analyte, each analyte should be injected separately to determine its retention time and to ensure that the impurities from one analyte do not had the same retention time as another analyte. For specificity, analyses of blank samples of the appropriate biological matrix (whole blood) should be obtained from at least six sources. Each blank sample was tested for interference using the proposed extraction procedure and chromatographic conditions.

System suitability

Five replicate injections of 5µg/ml strength Asiatic acid whole blood calibrated standard were injected under the same chromatographic conditions for system suitability test, peak area and retention time was determined.

Whole blood linearity

A series of whole blood sample solutions of Asiatic acid having concentration of 0.25, 0.5, 1.0, 2.5, 5, 10, 25, 50µg/ml were prepared by spiking the required volume of standard solution of Asiatic acid in 1ml drug free whole blood. 100microlitres of each of these solutions were injected using same chromatographic conditions. The chromatograms were recorded and the peak areas of the drug were calculated.

Precision and accuracy

Intra-day and inter-day precision was done at five different concentration liked 0.25, 2, 2.5, 20, 50µg/ml. Their % CV and % nominal were measured.

Percent extraction yield

The percent extraction efficiency was performed for 0.25, 5 and 50µg/ml concentration. For one sample 2 test tubes were taken. In one test tube required volume of calibrated standard solution was spiked to 1ml of blank whole blood and sample was extracted as per the developed extraction procedure. This sample was labeled as extracted. While in another test tube required volume of standard solution was added at reconstitution stage. This sample was labeled as unextracted. Both samples were injected in to chromatographic system.

Stability

At the different-different temperature and different-different time interval in specified area where the stock solution was kept and injected in to chromatographic system so their observation was gave knowledge about the stability of the active constituent in biologic matrix for particular time interval.



Pharmacokinetic study

Test product, Dose and Mode of Administration

Single oral dose of *Centella asiatica* L. 600mg/kg, 800mg/kg, 1000mg/kg powder (equivalent to 12.1mg Asiatic acid) with unlimited supply of drinking water in fasted state.

Procedure for collection of blood

All the rats were adult, healthy. Mean weight for the rats were 200-250gm respectively. No food was permitted for at least 10-12hrs before the administration of drug. A capillary was introduced in their eye and collect blood from retro orbital vein at fasting blood sample (0hrs pre dosing). After that 600mg/kg, 800mg/kg, 1000mg/kg dose of *Centella asiatica* extract were administered in form of suspension with prepared in to the gum acacia to different groups with respective dose. Two different groups were available in single dose of the drug and each group of different-different dose had 4 animals in it. Total 24 animals had been used in to the three dose containing groups while remaining 6 animals were also used for the blood sample collection. The blood was collected in tubes containing 0.1ml 10% EDTA. Post dose sampling times after drug administration were 0.5, 1, 2, 4, 8, 10 and 24hrs. Blood samples of 0.5, 2, 8 and 24hrs were collected from the cardiac puncture at that time organs collection like brain, heart, kidney, liver and homogenate it. The homogenate organs were collected in tubes containing 0.1ml toluene. Blood samples of 0 (pre dosing), 1, 4, 10hrs were collected from the retro orbital. For the blood samples were collected using two different methods on the each animal. Blood samples were stored frozen at $-20^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ with appropriate labels identifying subject numbers and time of blood collection. Pharmacokinetic parameter liked C_{max} , T_{max} , $T_{1/2}$, K_{el} , AUC which was statistically evaluated. Ethical approval had been taken from the R.C. Patel Institute of Pharmaceutical Education and Research, Shirpur.

RESULTS AND DISCUSSION

Specificity or selectivity

Each blank sample was tested for interference using the proposed extraction procedure and chromatographic conditions. The absence of peak in the blank whole blood at the retention time of standard indicates the specificity or selectivity of the method of analysis from whole blood.

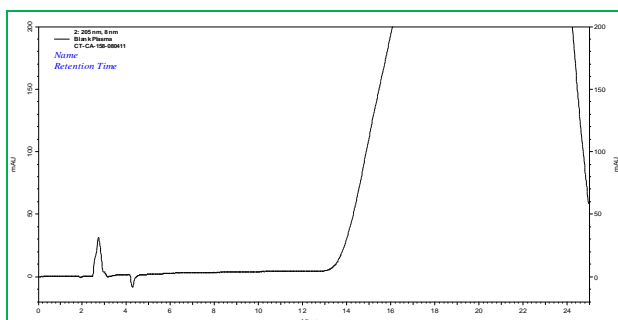


Figure 2: Chromatogram of blank solution

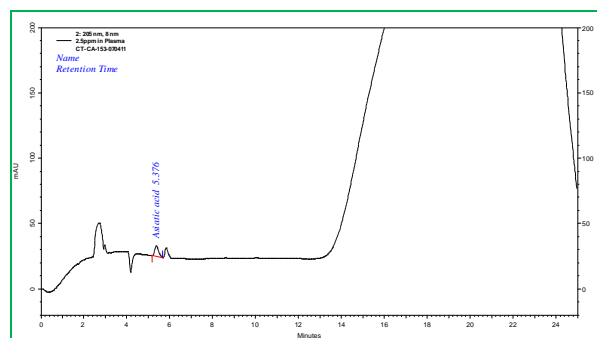


Figure 3: Chromatogram of spiked sample solution

System suitability

Standard Asiatic acid of $5\mu\text{g/ml}$ concentration was injected 5 times on the same day one by one. The precision and standard deviation were measured. Mean \pm S.D., percent Coefficient of Variation for the area was found to be 186882 ± 565.85 , 0.30; for retention time it was found to be 5.49 ± 0.04 , 0.69.

Linearity

The plot of concentration Vs area for the linear working range was depicted in Figure 4. The plot showed that a linear relationship exists in the given range of concentration. The coefficient of regression i.e. R squared was 0.9999, means that 99.99% of the variation in Y i.e. response of the analyte can be explained by the change in X i.e. concentration of the analyte. The correlation coefficient was a measure of the goodness of the fit of the calculated line to the sample data. The slope of the regression line found by calculation was 37941.32 and by graph was 38087.

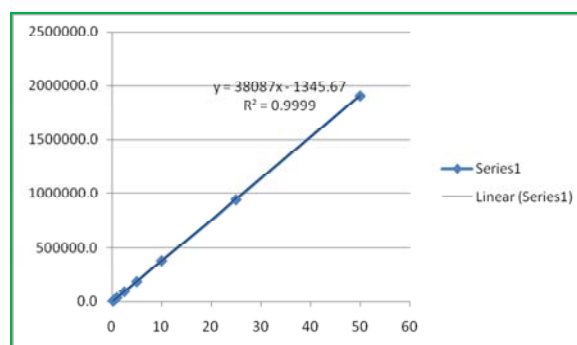


Figure 4: Linearity graph of Concentration Vs time

Sensitivity

Sensitivity was up to $0.1\mu\text{g/ml}$, which was LOD concentration and LOQ was $0.25\mu\text{g/ml}$.

Accuracy and precision

Within-series results

The coefficient of correlation for Asiatic acid varies from 0.9996 to 0.9998.

Precision (% CV) obtained at the $0.25\mu\text{g/ml}$, $2\mu\text{g/ml}$, $2.5\mu\text{g/ml}$, $20\mu\text{g/ml}$ and $50\mu\text{g/ml}$, concentrations were 4.25%, 1.58%, 1.06%, 1.15% and 1.68% respectively. The results were well within acceptance limit i.e. not more

than 15% for the lowest concentration and not more than 20% for all other concentration. Accuracy (% Nominal) obtained at the 0.25 μ g/ml, 2 μ g/ml, 2.5 μ g/ml, 20 μ g/ml and 50 μ g/ml concentrations were 105.41%, 101.13%, 100.09%, 104.98% and 101.29% respectively. The results were well within acceptance limit i.e. 80% to 120%.

Between-series results

The coefficient of correlation for Asiatic acid varies from 0.9991 to 0.9999.

Precision (% CV) obtained at the 0.25 μ g/ml, 2 μ g/ml, 2.5 μ g/ml, 20 μ g/ml and 50 μ g/ml concentrations were 4.03%, 1.50%, 1.73%, 1.57% and 1.96% respectively. The results were well within acceptance limit i.e. not more than 20% for the lowest concentration and not more than 20% for all other concentration. Accuracy (% Nominal) obtained at the 0.25 μ g/ml, 2 μ g/ml, 2.5 μ g/ml, 20 μ g/ml and 50 μ g/ml concentrations were 106.89%, 102.25%, 100.20%, 104.97% and 100.02% respectively. The results were well within acceptance limit i.e. 80% to 120%.

Percent extraction yield

The percent extraction yield obtained at 0.25, 5 and 50 μ g/ml. The percent extraction yield was found to be in the range of 73.72 to 74.34%.

Stability

The stability of Asiatic acid in biological matrix which was measured that demonstrates that the concentration of Asiatic acid was under the specified limit conditions even after the period of 14days.

Pharmacokinetic results

For the three different doses 600mg/kg, 800mg/kg and 1000mg/kg above given parameters were statistically determined their result was get significant which was mansion here.

For 600mg/kg result was T_{max} : 4hrs; C_{max} : 1.8 μ g/ml; $AUC_{(0-t)}$: 17.2 μ g/mlxhrs; $AUC_{(0-\infty)}$:23.4 μ g/mlxhrs; $T_{1/2}$:12.1hrs; K_{el} : 0.1hrs⁻¹.

For 800mg/kg result was T_{max} : 4hrs; C_{max} : 2.35 μ g/ml; $AUC_{(0-t)}$: 23.23 μ g/mlxhrs; $AUC_{(0-\infty)}$:24.82 μ g/mlxhrs; $T_{1/2}$:12.65hrs; K_{el} : 0.828hrs⁻¹.

For 1000mg/kg result was T_{max} : 4hrs; C_{max} : 2.9 μ g/ml; $AUC_{(0-t)}$: 29.13 μ g/mlxhrs; $AUC_{(0-\infty)}$:30.79 μ g/mlxhrs; $T_{1/2}$: 12.59hrs; K_{el} :0.857hrs⁻¹.

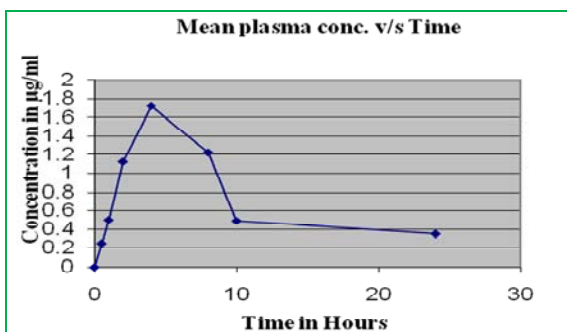


Figure 5: Concentration v/s time curve 600mg/kg

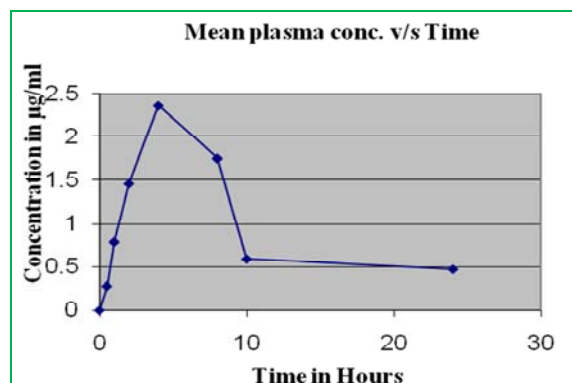


Figure 6: Concentration v/s time curve 800mg/kg

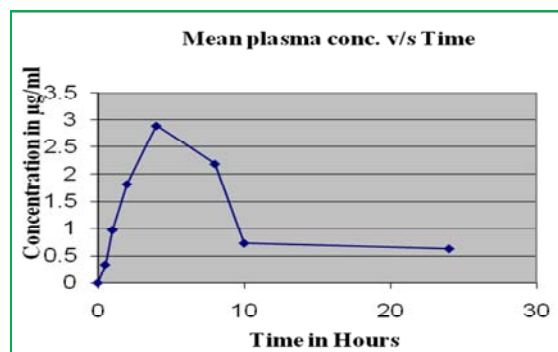


Figure 7: Concentration v/s time curve 1000mg/kg

CONCLUSION

Bioanalytical method was first developed then validated and concluded that method as standardized and validated was simple, reproducible, sensitive and specific for the determination of Asiatic acid from whole blood of animal. Hence this method can be applied to the study of pharmacokinetic parameters of Asiatic acid from whole blood of animal employing RP-HPLC as the method of analysis. An oral administration of the drug was detected up to 24hrs in the animals. It shows maximum concentration of drug at 4hrs which indicate their T_{max} and after that the concentration was decline. Result of Pharmacokinetic study showed that the Asiatic acid was rapidly absorbed by oral administration. We got good significant result of it so we said that it can be applied for *in vivo* study and their more pharmacological and clinical study.

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