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Extraction of Gallic Acid isolated from *Terminalia Chebula*, Analysis by Liquid Chromatography−Mass Spectrometry and its Pharmacokinetics in Mice & Rats

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A B S T R A C T

Gallic acid (GA) is an isolated secondary metabolite from *Terminalia chebula*, a common medicinal plant regularly used in the traditional preparation of several formulations in Indian system of medicine –Ayurveda. Extraction and processing of plasma samples containing GA from animals is a bit challenge due to its instability. Plasma samples were extracted with and without acidic environment and observed that hydrochloric acid addition has showed better extraction and recovery of GA. Later different formulations have shown very fast absorption and elimination half-life of GA in mice and rats.

Keywords: liquid chromatography−electrospray-ionization mass spectrometry, rat plasma, plasma protein binding; *Terminalia chebula*

A R T I C L E I N F O

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1. Introduction

Plant secondary metabolites are very good source for the discovery and development of active drug molecules in research since ages. Herbal medicines are well known for treating several ailments and diseases. These are used as therapeutic agents and are considered as Complementary and Alternative Medicines (CAM). *Terminalia chebula* (TC) is tree and its bark, fruits etc are well known in Indian system of medicine – Ayurveda, and is widely used for treating, digestion, constipation, inflammation, pain, etc.,

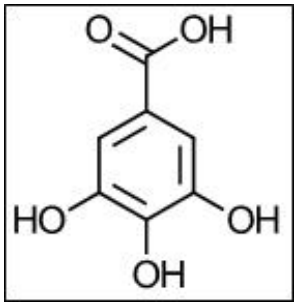

Figure 1: Gallic acid

T. chebula is commonly known as Harithaki and it is a main adjuvant in the preparation of 'Triphala'. It is used as a laxative in chronic constipation and also as a rejuvenator of the body. Recent studies revealed that *T. chebula* is possessing biological properties like, antioxidant, antibacterial, antifungal, antidiabetic, anticancer, antiulcer, wound healing etc [1]. The chemical constituents presented in the plant extract are mainly tannins, chebulic acid, chebulinic acid, chebulagic acid, ellagic acid, gallic acid etc [2-10]. In our recent article we presented the gallic acid (GA) extraction, isolation and identification and its antiviral activity against HIV strain 92HT599 [2]. GA has shown good inhibition against 92HT599 strain with IC50 value at 0.49 µM. Gallic acid is the major secondary metabolite observed in *T. chebula* during isolation and it is 18% in the aqueous extract. It was identified as free form, methyl or ethyl esters, or as gallate adduct. Due to the abundance of GA in *T. chebula* extract, the plants use in several medicinal uses can be attributed to GA.

Gallic acid (Fig.1) was isolated from *T. chebula* as described in our recent article [2]. Plant dried fruits powder was extracted with Methanol and subsequent column fractionation with hexane – chloroform - methanol combinations has yielded GA along with other secondary metabolites. Thus obtained GA was given orally to mice and rats to evaluate the pharmacokinetics (PK) using LCMS/MS. We did not observe much information about its pharmacokinetic information in the literature. There was published information about the extraction and the determination of GA in Rat and Rabbit plasma [6, 7]. We were not successful in reproducing the published rabbit plasma and rat plasma extraction analysis methods. There were issues with handling the plasma samples as GA is an acid and due to the pH of plasma. Hence we wanted to develop a more robust method to analyze GA for the evaluation of PK in rats and mice. Anticipating acidic compounds may form acyl glucuronide metabolites, we developed an extraction method using hydrochloric acid to stabilize the entire plasma system. In this paper we have not studied the metabolite identification but our method can be used to study about metabolite profiling also. Analysis of GA is challenging because of its complexity and general instability. In this article we evaluated the PK of gallic acid in rat and mice plasma using a simple and reliable LCMS/MS method. Our objective in this study is to develop a reliable method for extraction, and quantification by liquid chromatography−electrospray-ionization mass spectrometry (LC/ESI-MS) of GA isolated from *T. chebula*.

2. Materials and Methods

GA was obtained from Sigma, USA. Acetonitrile and dimethyl sulphoxide (DMSO) were purchased from Merck, USA. *Terminalia chebula* fruits were collected from Ayurvedic Departmental Stores, Hyderabad, India. Formic acid (FA), sulphuric acid (SA), phosphoric acid (PA), Sulphuric acid and hydrochloric acids (HCl) were purchased form Merck, USA. Mice, Rat, Dog, Monkey and Human liver microsomes and NADPH were purchased from Xeno biotech, USA.

1. Extraction of GA from *Terminalia chebula* fruits

The dried fruits of *Terminalia chebula* are powdered in an electrical grinder. 250 gm of the powdered fruit was taken in hexane (1000 mL) and stirred for 24 h at room temperature and the hexane layer was decanted to remove fatty acids, chlorophylls and the lipids. Remaining crude residue was macerated with 95% ethanol (1000 mL) for 48 h at room temperature. The ethanolic solution was filtered using Buckner funnel and flask attached to suction pump and the residue was extracted two more times each again with 95% ethanol (500 mL).

The combined extracts were concentrated under reduced pressure using a rotary evaporator at 50-55°C to obtain a crude residue of 148 gm. This residue was tested for organic compounds by using high performance liquid chromatography (HPLC) and Liquid chromatography–mass spectrometry (LCMS). Analysis of LCMS and HPLC confirmed the fruit extract of *Terminalia chebula* contains phenolic compounds as major constituents. The crude extract of *Terminalia chebula* showed 18% of of gallic acid by HPLC and the molecular weight was confirmed by LC-MS which was further confirmed by the coelution with purchased Gallic acid. The pure gallic acid was isolated following column chromatographic technique from the crude extract of *Terminalia chebula* by using 100-200 mesh silica gel [2].

2. Incubations with Liver Microsomes

Gallic acid (1 µM) was incubated in 1 mg/mL of pooled male mouse, rat, dog, monkey and human liver microsomes (Xeno Tech, USA) separatelyin 0.1 M phosphate buffer at 37ºC. After pre-warming the mixture for 5 min, reactions were initiated by the addition of NADPH (1 mM). Aliquots (40 µL) were taken at 0, 5, 10, 20, 40 and 60 min, and the reaction immediately terminated by adding into 200 µL of

acetonitrile. Samples were centrifuged and the supernatant fractions analyzed by LC-MS/MS with multiple reaction monitoring (MRM). All incubations were performed in duplicate. Propranolol was used as the positive control. The LCMS area response of the analyte for Time=0(control) was set to 100%, the relative decrease in MRM area intensity over time against that of the control (percent parent decrease) was used to determine the half-life ($t_{1/2}$) of GA in the incubation [11].

3. Plasma Protein Binding (PPB)

Plasma samples (of Human, Mice, Rat, and Dog at 200 µL each separately) were spiked with test compound (GA) at a concentration of 10 uM and added into the sample chamber of the RED divice. 350 µL of Phosphate buffer solution (PBS) was added into the buffer chamber of the device. This assay was conducted in duplicate using Propranolol as positive control. The test plate was sealed and incubated for 6h at 37 ^{o}C on an water shaker. After incubation, 100 µL of each chamber was transferred to a well of 96 well plate containing 100 µL of each species plasma, separately. Cold acetonitrile (500 µL) containing 10% hydrochloric acid was added to each sample to precipitate proteins. The samples were centrifuged, and the resultant supernatants were evaporated to dryness under stream of Nitrogen. The residue was reconstituted with 100 µL of mobile phase (20:80 of mobile phase A and B) and was transferred to vials for LC-MS-MS analysis [12].

4. Extraction of GA from Rat and Mice plasma

After thawing plasma samples at room temperature, a 250 µL of neat acetonitrile was added to 50 µL of plasma. This mixture was vortexed for 5 min and centrifuged at 9000g for 10 min. Thus obtained supernatant organic layer was transferred to a tube and evaporated to dryness under stream of Nitrogen gas in water bath at 35°C. This residue was dissolved in 200 uL of mobile phase and centrifuged for 2 min. A 50 µL of aliquot was injected on to the LC-MS-MS system for analysis. The same extraction was repeated with another set of 50 µL plasma samples by addition of a 250 µL of 10% acidic acetonitrile. In another set of experiments, plasma samples were collected in vials containing 10 µL of 10 % acid (FA, HCl, sulphuric acid and Phosphoric acid separately) solutions. These plasma samples were processed with the same procedure as mentioned above.

5. LC/ESI-MS Analysis

LC/ESI-MS analysis of GA isolated from *Terminalia chebula* was carried out using the Agilent 1200 LC system coupled to an Agilent 6460 Triple Quad mass spectrometer (Agilent, Santa Clara, CA, USA). The chromatographic conditions were optimized as to be compatible for analysis by the electrospray triple Quadruple instrument. The chromatographic separation was carried out using a (100 × 4.6 mm, 3.5 µm) Eclipse Plus C18 column at 30 ^{o}C. The GA was then eluted from the column using isocratic program of 20% A and 80% B in 4 min at a flow rate of 600 µl/min. Mobile phase A consisted of 10 mM ammonium formate while mobile phase B consisted of methanol. The tandem mass spectrometer was having ESI source, and run with Mass hunter B.06 software (Agilent). The MS system operated with negative ion mode with precursor to product ion transition m/z 169.07 to 125.01 for GA. The mass spectrometric source and ion optic conditions were optimized to get maximum sensitivity as follows; capillary voltage 4000 v, heated gas temperature 350°C, gas flow and nebulizer pressure at 12, and 60 arbitrary units respectively. Collision energy was 15v and dwell time was 200 msec.

6. Formulation Preparations and Collection Time

GA was dosed as a solution in different formulations (F1 – F4) as a single dose via intravenous bolus (3 mg/kg), oral gavage (po; 30 mg/kg) administration. The dose volumes of GA in the IV and PO solutions dosed to mice and rats were given at 1mL/kg and 10 mL/kg respectively. Formulation 1 (F1) is prepared by dissolving 1% CMC in water followed by GA to give 3 mg/mL solution. Formulation 2 (F2) was prepared by addition of GA to a 5% Solutol and 10% PEG 400 at 60-70 ^{o}C followed by addition of remaining 85 % water to get a clear solution. Formulation 3 (F3) was prepared by addition of GA to a 10% PEG 400 at 60-70 ^{o}C followed by addition of remaining 90 % water to get a clear solution. Formulation 4 (F4) was prepared by addition of GA to a 10% beta-Cyclodextrin at 60-70 ^{o}C followed by addition of remaining 90 % water to get a clear solution. Blood (for preparation of plasma) samples (50 µL) were collected in eppendorf vials containing 10 µL of 10% HCl. The time points were predose, 0.08, 0.25, 0.5, 1, 2, 4, 8, and 24h for IV dosing and predose, 0.5, 1, 2, 4, 8, and 24h for PO in both mice and rats.

7. Animal PK studies

Sprague Dawley (SD) rats (32 adult males), and Swiss albino mice (32 adult males), were bred in house and 4 animals were used for each formulation. 16 animals were used for Group 1 (with acid addition to plasma sample) and 16 animals for Group 2 (without acid addition) to study acidic pH effect on the extraction process. Animals were individually identified by tail markings and were acclimated to the study environment for 24 h prior to dose administration. Animals were individually housed in suspended wire caging, and were kept on a 12h/12h light/dark cycle except when interrupted for study procedures. Average room temperature was regulated in the range 20°C±2, average relative humidity of 30-70%. Animals were fed Harlan Certified Rodent Diet Meal food ad libitum, prior to dose administration, and had access to water ad libitum. The observed time and concentration data was analyzed by using Phoenix 2.0 software.

3. Result and Discussion

Extraction of GA from the plasma samples was the key step for successful analysis. However, GA extraction from plasma was very low due to the instability or reactivity or not having appropriate pH of the plasma. GA is an organic acid with high polarity. We tried to extract plasma samples with organic solvents (ethyl acetate, acetonitrile and dichloromethane) and with acidified (Formic acid, phosphoric acid, sulphuric acid and hydrochloric acid) organic solvents. The published procedure to extract GA in presence of phosphoric acid could not able to be reproducible. In another procedure with sulphuric acid

extraction seems to be working but we wanted to minimize the usage of acid volume and simplify the procedure. So we tried different acids like, formic acid, phosphoric acid, sulphuric acid and hydrochloric acid along with water and phosphate buffer. The LCMS/MS analysis of the extracted plasma samples revealed that the acidified acetonitrile with hydrochloric acid has shown above 95 % extraction efficiency (Table 1).

The plasma extraction with acetonitrile has shown no GA peak in LC-MS-MS analysis. Then the extraction procedure was repeated with acidified acetonitrile, but again GA peak was not detected. Hence, this extraction and analysis procedure was performed in two different ways. The rat and mice plasma samples were collected with and without hydrochloric acid addition to the collection vials. Plasma samples were extracted with the procedure mentioned in sec 2.1. These samples were analyzed by LCMS/MS for GA (see Figure 3 for spectral data). Analysis indicated that the acidified samples (Group1) have shown GA peak but not the unacidified plasma samples (Group2). This indicated that GA is either not stable in the unacidified condition or it has certain binding property to the tissues or to the plasma proteins in the circulatory system. The plasma protein binding (PPB) experiment with GA was shown to be highly bound to plasma (> 99 %) across all species (Human, Dog, Rat, Mice) tested.

The GA was dosed to Rats and mice with 4 different formulations. Formulations were prepared freshly and dosed to mice and rats orally with described vehicles (as mentioned in Table 2) at 30 mg/kg. Plasma concentrations dosed PO were analyzed at 30 min, 1 h, 2h, 4h, 8h, and 24h time points using LCMSMS were processed with Phoenix software for the PK data (Table 3). In mice, the absorption and elimination rate were very fast and the compound (GA) highest PK of formulation F4 was observed with 1.4 µg/mL Cmax at 30 min time point with an exposure of 0.963 µg.h/mL. All the formulations have shown the same trend of fast absorption and elimination rates in both mice and rat species. Out of all, formulations F1 and F4 in mice and F4 in rats have shown better PK parameters. In rats also similar kind of trend in PK was observed. Both absorption and elimination rates were very fast and the exposures ranged from 0.482 to 0.746 µg.h/mL in the observed four formulations. Suspension formulation, 1% CMC has shown better PK parameters among others in both rat and mice. We still need to explore for a better and suitable formulation which is more specific for high polar acidic compounds when dosed in both species.Liver microsome stability studies showed that GA is a stable compound across all species (Mice, Rat, Dog, Monkey and Human) tested and the results are shown in Figure 2. Further in vitro and in vivo studies with GA are ongoing. Our extraction method was established for analysis of GA in mice and rat plasma samples after oral administration of different formulations for pharmacokinetic studies. To our knowledge, this is the first time report of mice and rat pharmacokinetic data in the literature.

Table 1: Percent recovery of Gallic acid with different solvent systems

System	% Recovery
Water	1
Phosphate Buffer	1
Hydrochloric acid	95
Formic acid	10
Phosphoric acid	12
Sulphuric acid	18

Table 2: Formulation details used for dosing animals

Formulation	Composition
Formulation 1	1% CMC
Formulation 2	5% Solutol and 10 % PEG400
Formulation 3	10 % PEG400
Formulation 4	10 % β-Cyclodextrin

Table 3: Pharmacokinetics data of GA in Rats and Mice with different formulations

Species	Formulation	Cmax (ug/mL)	Tmax (hr)	Half-life (hr)	AUC0-t (µg.hr/mL)
Mice	1% CMC (F1)	1.053	0.875	NA	0.950
	% solutol+10% PEG (F2)	0.685	0.500	NA	0.704
	10% PEG (F3)	0.885	0.750	2.320	0.927
	10% β-cyclodextrin (F4)	1.421	0.500	NA	0.963
Rats	1%CMC (F1)	1.156	0.500	5.627	0.746
	% solutol+10% PEG (F2)	0.452	0.625	2.848	0.580
	10% PEG (F3)	0.251	1.375	2.527	0.641
	10% β-cyclodextrin (F4)	0.32	0.63	2.624	0.482

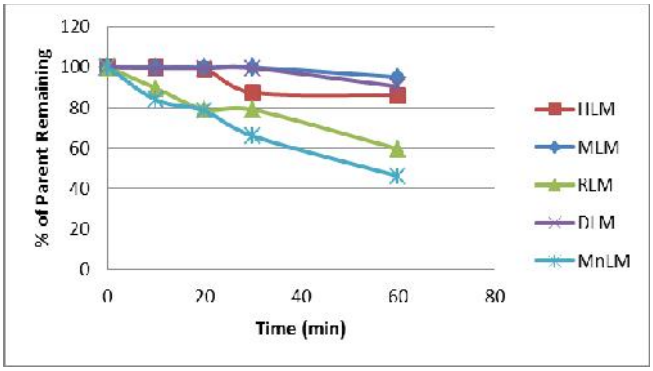

Figure 2: Liver microsome Stability of Gallic acid

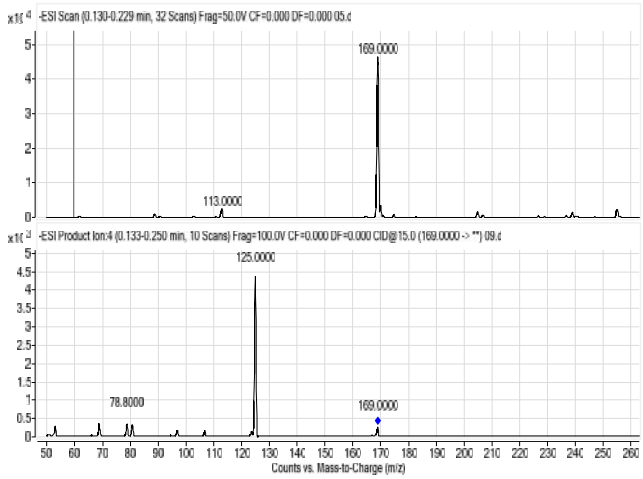

Figure 3: Mass spectrum of GA (negative electrospray) isolated from *Terminalia chebula* extract at 1.9 min retention time.

4. Conclusion

This paper describes a relatively easy GA extraction procedure from plasma samples and its analytical procedure and detection by LC/ESI-MS. The percent recovery is good and reproducible with hydrochloric acid treated plasma samples during solvent extraction procedure than those of the other tested acids. Though the stability of GA is low at room temperature, addition of 10% hydrochloric acid has increased the stability of it after immediate addition to plasma samples. Liquid chromatography with Eclipse plus column provided good separation and peak properties for Gallic acid using a mobile phase that is compatible with negative ion electrospray mass spectrometric detection. This procedure is suitable for analysis of the naturally occurring GA from plant resources. Liver microsome incubations with GA have indicated that it is stable across all species. Plasma protein binding studies indicated that GA is very highly bound compound across all species tested. Both in mice and rats, absorption and elimination of GA were very fast. Formulation F1, has shown better PK properties amongst all the other formulations.

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6. References

1. Ruwali Pushpa, Rai Nishant, Kumar Navin, Gautam Pankaj Anti-viral potential of medicinal plants – an overview. *Int. Res. J. Pharm.* **2014**, 4 (6), 8-16.
2. Sravan Kumar Reddy Theepireddy, Ramakrishna Chinthala, L. Vaikunta Rao, V. V. Kurisetty, Rathanakar Reddy Kura, Mallikarjuna Rao Duvvada. The isolation, characterization and quantification of gallic acid from the fruit extract of *Terminalia chebula*. *Int J Med and Pharm Res*, **2015**, 3(2): 983–988.
3. R. Rathinamoorthy, G.Thilagavathi. *Terminalia Chebula* - Review on pharmacological and biochemical studies. *International Journal of Pharm Tech Research*. **2015,** 6(1):97-116.
4. Yadav Babita, Keshipeddi Sandhya Rani, Bhat Sulochana, Singh Mamta. A perspective study of Haritaki. *IJRAP*, **2011**, 2 (5): 1466-1470.
5. Sravan Kumar Reddy Theepireddy, Ramakrishna Chinthala, L. Vaikunta Rao, Santhosh Vishwakarma, Rathanakar Reddy Kura, Mallikarjuna Rao Duvvada. Anti-HIV activity of *Terminalia chebula* crude extracts and gallic acid. *Int J Pharm and Tech*, **2015**, 6(1): 6346–6354.
6. Xinfeng Zhao, Weijin Zhang, Shanshan Kong, Xiaohui Zheng, Jianbin Zheng, Runju Shi. A valid assay for the pharmacokinetic study of gallic acid from *Choerospondiatis fructus* in rabbit plasma by LC/MS/MS. *Journal of Liquid Chromatography & Related Technologies.* **2007**, 30(2): 235-244.
7. Rui Song, Lei Xu, Zunjian Zhang,Yuan Tian, Fengguo Xu, Haijuan Dong. Determination of Gallic Acid in Rat Plasma by LC-MS-MS. *Chromatographia*. **2010**, 7: 1107-1111.
8. Vazirian, Mahdi, Khanavi, Mahnaz, Amanzadeh, Yaghoub, Hajimehdipoor, Homa. Quantitation of Gallic acidin in fruits of three medicinal plants. *Iran. J. of Pharm. Res.* **2011**, 10(2): 233-236.
9. Md. Golam Kibria, Md. Musarraf Hussain. *In-vitro* cytotoxic effect of methanolic crude extract of *Terminalia chebula*. *Int. Res. J. of Pharm.* **2012**, 3(9): 94-95.
10. Ravikanth Veluri, Rana P. Singh, Zhengjie Liau, John A. Thompson, Rajesh Agarwal, Chapla Agarwal. Fractionation of grape seed extract and identification of gallic acid as one of the major active constituents causing growth inhibition and apoptotic death of DU145 human prostate carcinoma cells. *Carcinogenesis*, **2006**, 27(7): 1445–1453.
11. E. Kantharaj, An Tuytelaars, Pascale E. A. Proost, Zuleyha Ongel, Harrie P. van Assouw and Ron A. H Simultaneous measurement of drug metabolic

stability and identification of metabolites using ion-trap mass spectrometry. J. Gilissen. *Rapid Commun. Mass Spectrom*. **2003**, 17: 2661–2668.

12. Nigel j. Waters, Rachel Jones, Gareth Williams, Bindi Sohal. Validation of a rapid equilibrium dialysis approach for the Measurement of plasma protein binding. *Journal of Pharmaceutical Sciences*. **2008**, 97(10): 4586–4595.