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PHARMACOKINETIC EVALUATION OF GENTISIC ACID IN RATS: ORAL VERSUS INTRAVENOUS ADMINISTRATION

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ABSTRACT: Gentisic acid (2, 5-dihydroxybenzoic acid) is a diphenolic compound, present abundantly in natural sources. It possesses several pharmacological activities such as nephroprotective, hepatoprotective, neuroprotective, *etc*. However, the available literature on oral absorption of gentisic acid is scarce. Thus, the aim of the present study was to determine the pharmacokinetic parameters and bioavailability of gentisic acid. A single dose *in-vivo* pharmacokinetic study was conducted in rats. Gentisic acid was administered at 50 mg/kg, either orally or by intravenous route. Blood samples were withdrawn at designated time intervals (0.083, 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h post-dose), and plasma was separated. A method for the evaluation of gentisic acid in the plasma was developed and validated. The plasma levels of gentisic acid were determined by HPLC using the validated method. The C_{max} for oral administration was 312 µg/mL, and T_{max} was at 0.083 h. The plasma exposure AUC_{last} values for oral and i.v route of administration were comparable (Oral: 765 h*µg/mL; I.V.: 770 h*µg/mL), indicating similar bioavailability. The relative bioavailability for oral dose was found to be 99.35%. The results suggest that gentisic acid is absorbed through the oral route of administration and shows bioavailability comparable to intravenous administration.

INTRODUCTION: Gentisic acid (2, 5-dihydroxybenzoic acid) is a diphenolic compound and a derivative of benzoic acid with pKa=2.95. It is a metabolite of aspirin and a natural product from the root of the genus *Gentiana* [1]. Gentisic acid is present abundantly in natural sources. The plant sources include *Gentiana* species, grapes (*Vitis vinifera)*, citrus fruits, red sandalwood (*Pterocarpus santalinus)*, Jerusalem artichokes (*Helianthus tuberosus*), *Hibuscus rosa-sinensis*, olives (*Olea europaea*), and sesame (*Sesamum indicum*).

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It is also present in fruits such as avocados, batoko plum, kiwi fruits, apple, bitter melon, blackberries, pears, and in medicinal herbs such as Madagascar rosy periwinkle and is also a component of a wine. In addition to plants, gentisic acid is also produced by mushrooms, such as *Polyporus tumulosus* and *Penicillium patulum.* As mentioned earlier, gentisic acid is a metabolite of salicylates and is excreted in urine after ingestion of salicylates [1, 2]. However, gentisic acid is reported to be present in the plasma of individuals even when there has been no intake of salicylates, indicating a dietary source of gentisic acid-containing fruits and vegetables [3].

Epidemiological findings have revealed a positive relationship between consumption of a diet rich in phenolic acids such as gentisic acid and prevention of various ailments [4].

Gentisic acid has demonstrated various pharmacological activities, namely, antioxidant, anti-carcinogenesis, hepatoprotective, antimicrobial, analgesic and anti-inflammatory, neuroprotective, cardioprotective, nephroprotective, muscle relaxant, and skin-lightening [5]. Gentisic acid has been explored in our lab for its protective activity in diabetes-induced nephropathy in rats (unpublished data). However, one single paper from 1953 is available on the pharmacokinetics of gentisic acid [6]. Therefore, the present study aims to determine the pharmacokinetic parameters and bioavailability of gentisic acid.

MATERIALS AND METHODS: Gentisic acid 98% was procured from Sigma-Aldrich, USA. All the solvents used for the analysis were of HPLC grade (SD Fine Chemicals Ltd., Mumbai, India).

Animals and Treatment: Healthy male Wistar rats with a bodyweight of 200-250 grams were procured from the National Institute of Biosciences, Pune, India. The rats were housed in the animal house of the Institute of Chemical Technology, Mumbai (Reg No: CPCSEA/1999/81). Animals were fed with standard commercial laboratory feed from Nutrivet Ltd., India, and supplied with purified water *ad libitum*. The study was conducted in accordance with a protocol approved by Institutional Animal Ethics Committee (ICT/IAEC/2016/P05). The rats were acclimatized for 7 days prior to experiment. The animals were divided into 2 groups of 6 animals each as follows:

Group 1: Gentisic acid 50 mg/kg, intravenous administration

Group 2: Gentisic acid 50 mg/kg, oral administration

Prior to dosing, rats fasted overnight for 12 hours. Gentisic acid (GA) was dissolved in water and dosed to rats as mentioned.

A series of 9 blood samples were collected from each rat (n=3/time point) at 0.083, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h post-dose. At each time point, around 0.3 mL of blood was taken from retro-orbital plexus under isoflurane anesthesia and transferred into labeled tubes pre-coated with anticoagulant (4 mM K_2EDTA per ml of blood). The blood samples were mixed by manual inversion and kept on ice until centrifuged. Plasma samples were separated by centrifugation of the blood samples within 1 h of the collection at 5000 rpm for 10 minutes at 4 ºC. The separated plasma samples were transferred into labeled poly-propylene tubes and were stored at -80 °C until bioanalysis.

Bioanalysis:

HPLC Method Development for Gentisic Acid: A HPLC system of JASCO Corporation was used for the analysis. HPLC was equipped with an auto sampler and photodiode array detector system and an electrochemical detector. The chromatogram was analyzed by JASCO ChromNAV version 1.19.01 software. In house method was developed using stationary phase C18 column (4.6 mm × 250 mm; 5 um) by Jasco corporation with mobile phase composition of Acetonitrile and 0.1% ortho-phosphoric acid buffer pH adjusted to 2.5 with triethylamine (50:50), operated at 1ml/min flow rate.

Preparation of Stock Solution, Internal Standards and Test Samples: Gentisic acid stock solution (1 mg/mL) was prepared by dissolving 2 mg of gentisic acid in 2 mL of the calibrated volumetric flask using 50% acetonitrile in HPLC grade water. To aid the quantification of gentisic acid, ethyl ferulate was used as an internal standard. In an identical approach, 2 mg of internal standard (IS) was dissolved in 2 mL of a calibrated volumetric flask with 50% acetonitrile in HPLC grade water. Two different stock solutions were prepared for calibration of standards. A series of working solutions containing both gentisic acid and ethyl ferulate (50, 100, 150, 200, 250, and 50, 100, 150, 200, 250 µg/mL, respectively) were made by diluting the intermediate stock solution with 50% acetonitrile in HPLC grade water. An internal stock solution of 500 µL was added to a 25 mL volumetric flask, and the volume was made up with 50% acetonitrile in HPLC grade water to achieve a concentration of working solution of 20 µg/mL.

Sample Preparation: Samples were prepared as per guidelines available in the literature [7]. For rat plasma sample analysis, we prepared calibration curve standards and test samples by spiking 2.5 µL of spiking solution into 47.5 µL of interference-free blank plasma in pre-labeled vials, followed by

vortex to mix the samples. To aid the quantification of gentisic acid, we added 20 µL of internal standard working solution of ethyl ferulate to all tubes except for the standard blank sample, using a micropipette followed by vortex to mix. For the preparation of the blank sample, IS was replaced with 20 µL of 50% acetonitrile in HPLC grade water. Protein precipitation in plasma was carried out by adding 400 µL of precipitation solution (90% Acetonitrile with 1% acetic acid) to all tubes and vortex to mix for 5 min. All the quenched samples were centrifuged for 5 min at 3200x g and at set temperature of 4 °C. Subsequently, aliquot of the supernatant was transferred into HPLC inserts kept in 1 mL vials and cap with polyethylene plugs. 50 µL of the supernatant was injected to the HPLC column for quantitative analysis.

Analytical Conditions for Quantification of Gentisic Acid: The following conditions were maintained for the analysis of Gentisic acid

Column: Hypurity, C18 [250 × 4.6 mm, 5 µ Thermoscientific].

Mobile phase: Acetonitrile and 0.1% ortho-phosphoric acid buffer pH adjusted to 2.5 with triethylamine (50:50).

Flow rate: 1.0 ml/min.

Injection volume: 50 µl

Run time: 12 min

Detection wavelength: UV PDA detector at wavelength of 296 nm.

Temperature: 28º

Retention time: Gentisic acid (3.3±0.2 min).

Standard Curve Generation for Gentisic Acid: A calibration curve for gentisic acid was constructed using five samples covering a total range (50 - 250 µg/mL). The calibration curves were constructed with area ratios of analyte peak area and IS peak area using the least-squares linear regression model $y = mx + c$, where y denotes the observed area ratio, m the slope and c the intercept respectively, The acceptance criteria for calibration curve for the coefficient of determination (r^2) was >0.97. The acceptance criteria for each standard was ± 20%.

Pharmacokinetic Data Analysis: Non-compartmental analysis was used to determine the pharmacokinetic parameters of gentisic acid in rats. The area under the plasma concentration-time curve from time zero to the last quantifiable concentration (AUC_{last}) was calculated by the linear trapezoidal rule. The peak plasma concentrations (C_{max}), time to reach the peak plasma concentration (T_{max}), and time of the last quantifiable plasma concentration (T_{last}) were determined.

Bioavailability (BA) and Effective Dose (ED) of Gentisic Acid: Bioavailability is defined as the fraction of gentisic acid absorbed via oral route compared with *i.v.* route of administration, or the amount of drug available at the site of action. The bioavailability of gentisic acid was calculated using the following equation [8, 9]:

BA = (GA) [AUClast] oral × (GA) [Dose] i.v. / (GA) [AUClast] i.v.× (GA) [Dose] oral

The dose normalization factor is excluded in the above equation as the dose administered through oral and *i.v.* route was same.

After establishing the bioavailability of gentisic acid, we calculated the effective dose of gentisic acid based on the following equation[8, 9]:

Effective Dose of GA = Bioavailability of GA × Dose administered of GA

RESULTS AND DISCUSSION: In this study, we have developed a robust and rugged quantitative HPLC bioanalytical method capable of simultaneous analysis of gentisic acid along with ethyl ferulate. Currently, no HPLC method is available for the qualitative and quantitative analysis of both gentisic acid and ethyl ferulate. The current method involves quantification using Photodiode array detector and electrochemical detector, which ensures low sample volume for the determination of gentisic acid.

Calibration Curves of Gentisic Acid: The calibration curve of gentisic acid was prepared at total five different concentrations (50, 100, 150, 200, 250 µg/ml) of standard gentisic acid in rat plasma. A calibration curve was obtained by plotting AUC values (Y-axis) against concentration (X-axis). **Fig. 1** shows the graph and AUC for the standard concentrations.

The curve yielded the equation as y = 304.1x-7057.7 with R^2=0.99. The Standard chromatograms showing both gentisic acids and IS (ethyl ferulate) are displayed in **Fig. 2**.

Sr No	concentration (µg/ml)	AUC
1	50	9903
2	100	22542
3	150	37879
4	200	50529
5	250	71935

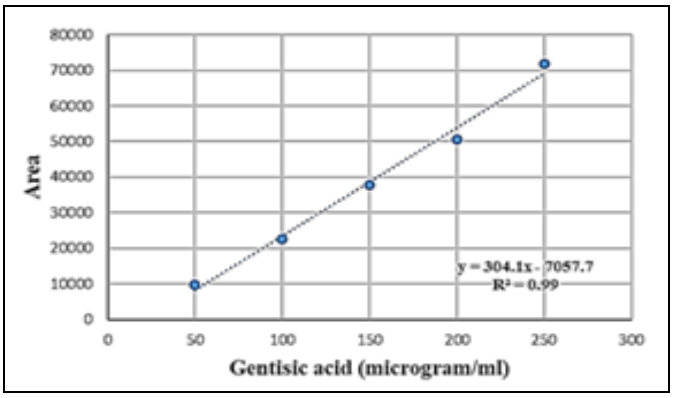

FIG. 1: AUC AND STANDARD CURVE FOR VARIOUS CONCENTRATION OF GENTISIC ACID (50- 250 µg/ml)

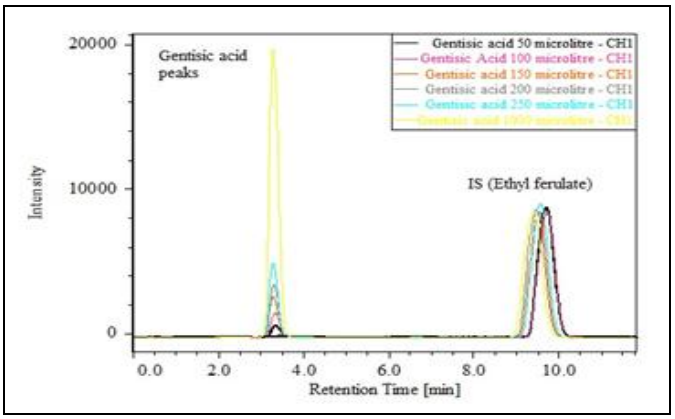

FIG. 2: STANDARD CONCENTRATION CHROMA-TOGRAMS OF GENTISIC ACID (50-1000 µg/ml)

Bioavailability (BA) and Effective Dose (ED) of Gentisic Acid: In our study, we determined the bioavailability of gentisic acid by comparing the oral route with *i.v.* route of administration. The BA of gentisic acid was found to be 0.99 or 99.3% of the administered gentisic acid. The effective dose calculated on the basis of BA was 49.67 mg of given 50 mg/kg.

Pharmacokinetic Analysis: For the pharmacokinetic analysis; Mean composite plasma concentrations (n=3 rats/time point) were used to determine the pharmacokinetic parameters of gentisic acid using the non-compartmental analysis module (extra-vascular and IV bolus) of validated Phoenix® WinNonlin® software (version 8.0). The plasma concentrations that were below the limit of quantification were considered as "zero" for mean concentration calculations. Concentration values below the LLOQ (lower limit of quantification: 28 µg/mL) were treated as '0' for descriptive statistics and pharmacokinetic analysis. The mean plasma concentration-time profile of gentisic acid after oral and i.v. administration is shown in **Fig. 3,** and their pharmacokinetic parameters are summarized in **Table 1**.

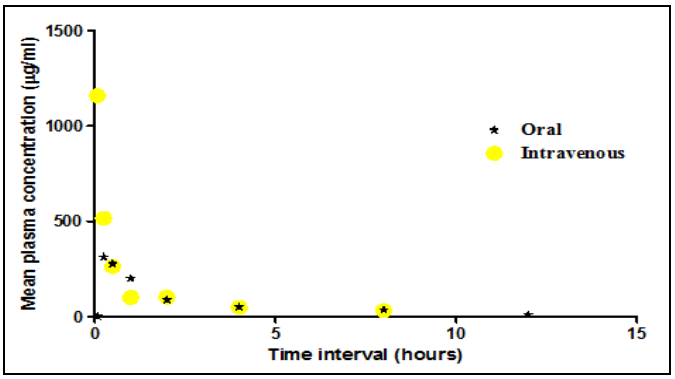

FIG. 3: LINEAR PLOT OF MEAN PLASMA CONCEN-TRATION-TIME PROFILES OF GA FOLLOWING i.v. AND ORAL ADMINISTRATION

TABLE 1: PHARMACOKINETIC PARAMETERS OF GA AFTER i.v. AND ORAL ADMINISTRATION TO RATS

Dose	Route	T_{max} (h)	C_{max} (µg/mL)	AUC_{last} (h*µg/mL)	C_{last} (µg/mL)	T_{last} (h)	$T_{1/2}$ (h)
50 mg/kg	i.v.	0.0830	1060	770	31.0	8.00	3.76
50 mg/kg	oral	0.250	312	765	9.33	12.0	4.64

The dose of 50 mg/kg was selected based on the literature available for various *in-vivo* pharma-cological actions of gentisic acid. Gentisic acid was most effective at a dose of 50 mg/kg or higher dose in rats [5]. Unpublished data from our laboratory has also shown that gentisic acid at 50 mg/kg prevents oxidative stress in diabetic rats.

As mentioned previously, the available literature on pharmacokinetics of gentisic acid is scarce. A report published in 1953 by Clarke and Mosher has studied the metabolism of sodium gentisate and gentisic acid in human subjects [6]. In this procedure, the levels of gentisic acid are determined using a colorimetric method, which relies upon the formation of blue color by the reaction of gentisic acid with an acid solution of ferric and ferrous chlorides. However, it is now known that colorimetric procedures for quantification are less accurate and sensitive as compared to currently available highly efficient methods such as HPLC. Therefore, in the present study, gentisic acid was quantified using a validated HPLC method.

After oral administration, gentisic acid was rapidly absorbed with a maximum plasma concentration (C_{max}) of 312 µg/mL at 15 min. Clarke and Mosher have also observed that upon oral administration of sodium gentisate to human subjects, a measurable amount of gentisate appeared in the bloodstream after 15 min, however, the result was not quantified [6].

The plasma exposure AUC_{last} values for oral and *i.v* route of administration were comparable (Oral: 765 h*µg/mL; I.V.: 770 h*µg/mL), indicating similar bioavailability. The relative bioavailability for oral dose was found to be 99.35%.

The time of the last quantifiable plasma concentration was 12 h and 8 h, for oral and *i.v*. administration respectively, indicating that the *i.v*. dose undergoes clearance at a rapid rate from the body. Gentisic acid is a small water-soluble molecule, mainly excreted in urine as conjugates of ethereal sulphates, glucuronides, or glycine derivates, with less than 1% being excreted in the bowel [6]. This explains the overlapping mean plasma concentration values for *i.v* and oral route of administration **Fig. 3**, as well as rapid excretion *via i.v* route of administration.

CONCLUSION: The pharmacokinetics of gentisic acid on oral and intravenous administration has been demonstrated in rats. The results suggest that gentisic acid is absorbed through the oral route of administration and shows bioavailability comparable to intravenous administration. The results indicate the effective oral dose to be 49.67 mg/kg upon oral administration of 50 mg/kg, suggesting that all the administered drug may be available for the desired pharmacological action to treat the intended disease.

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CONFLICTS OF INTEREST: None

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