Comparative Pharmacokinetics of Free and Liposome-Encapsulated Catechin after Intravenous and Intraperitoneal Administration

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ABSTRACT: Our previous studies demonstrated the efficacy of liposome-encapsulated catechin on the reduction of liver toxicity in rats with the injection of carbon-tetrachloride in vivo. In this investigation, pharmacokinetics of catechin after the injection of solution and liposomal catechin formulations developed in our laboratory were studied in rats. Two different formulations, one multilamellar vesicles (MLVs) intended for sustained release and other small unilamellar vesicles (SUVs) intended for targeted release were used. MLVs were injected by IV route while SUVs by IV route. Pharmacokinetic properties of liposome-encapsulated and free catechin were compared. Concentrations of the drug in plasma were determined by HPLC. PK analysis was performed using either WINNONLIN or KINETICA and both, compartmental and non-compartmental parameters were evaluated. Results indicated that, when catechin liposomal formulations were administered by IV and IP routes, mean residence time (MRT), half-life (t1/2) and the volume of distribution (Vd) were higher (P<0.05), and clearance (CL) was lower (P<0.05) than in the case of the free form. When the formulation was administered by IP route, the area under the curve (AUC) and the time to peak concentration (tmax) were significantly higher (P<0.05), and maximum concentration (Cmax) of catechin was lower than those of the free form. The results obtained in the present study showed that liposome encapsulated catechin provides the effective and prolonged plasma concentration after IV and IP administration.

KEY WORDS: catechin, cirrhosis, cancer, intravenous, liposomes, sustained release, pharmacokinetics

Introduction

Our current study deals with pharmacokinetic investigations on catechin (Fig. 1), one of the flavan-3 ols belonging to catechins family of phytochemicals, and its liposomal formulations. Catechin was first isolated from plant extract catechu, Acacia catechu – Leguminaceae from which it derives its name and has been mentioned in several Ayurvedic and other ancient medical texts for its therapeutic role (Lahiri and Bhide, 1993). Recent research demonstrated a variety of pharmacological and therapeutic uses for this drug which include reduction in oxidative stress, hepatoprotection, improving memory, reducing chronic fatigue, colorectal cancer, collagen stabilization, etc (Zaveri, 2006). Most of these actions have been attributed to its antioxidant activity. Currently our group is focusing on the development of new treatments and new delivery systems in cirrhosis, a disease which needs hepatoprotection. Currently, there is no proper treatment for this disease and people rely on liver transplantation or Liv 52, only. Although several synthetic and natural agents are in preclinical research to treat cirrhosis, for a variety of known reasons, we, in our laboratory, are currently conducting preclinical investigations on some natural products, viz., piperine, silymarin, catechin, curcumin, glycerrhizin, etc. for their application against cirrhosis, especially applying new nanotechnology based delivery systems. We previously reported piperine as a hepatoprotective agent (Bonepally et al., 2008). The second molecule we are investigating on these lines is catechin. In cirrhosis, several liver cells are affected (Albanis and Friedman, 2006). The mammalian liver consists primarily of hepatocytes, kupffer cells (KC), liver sinusoidal endothelial cells (LSEC) and stellate cells. Of these KC and LSEC are known to take up particles and thus higher intracellular levels which may be the prerequisite for effective treatment of several diseases can be achieved by having drugs encapsulated in particles and targeting the respective cells. Fortunately, oxidative stress in KC and LSEC was found to be main initiator of liver cirrhosis, although several other pathways are also involved. In the light of these issues, therapy with the natural antioxidant catechin can be enhanced by having drugs administered in the form of liposomal particulates. As this aim persists, we developed a catechin liposomal formulation and demonstrated its efficacy in a CCl₄ treated liver cirrhosis model (Ganji and Aukunuru, 2008). On the other hand, the purpose of this study is to investigate the pharmacokinetics of liposomal catechin in a rat model. The data from this study adds knowledge to our quest to improvise therapy against cirrhosis and also adds to the existing knowledge in this area. Liposomes can over come the solubility limitations of drugs, protect it in the active confirmation, reroutes the drug from sites of toxicity, prolongs circulation time, provides sustained release and in this case, may increase KC and LSEC accumulation via phagocytosis and endocytosis (Ogawara et al., 1999). The

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results from this study have implications in several other diseases where in catechin is useful and also adds information to a currently popular subject area called tea catechins.

![Fig. 1 Catechin.](image)

Catechin along with EGCG, EGC, ECG belongs to tea catechins. Many laboratory studies have demonstrated the inhibition of tumorigenesis by tea and tea polyphenols in different animal models including mouse skin, mouse lung, rat and mouse esophagi, mouse duodenum, rat small intestine, rat colon, and rat and mouse liver (Fang et al., 2007). However, epidemiological studies are inadequate. Even in studies with animals, mechanistic understanding of the inhibitory effect of tea on tumorigenesis is hampered by insufficient information regarding absorption, distribution, metabolism and elimination of the effective components of tea. It is believed that most of the cancer-inhibitory activity of tea is due to polyphenols present in tea. However, there are very scanty reports regarding pharmacokinetics of these tea catechins. The data from this study thus also sheds light regarding the pharmacokinetics of catechin, a major tea catechin. No reports regarding pharmacokinetics of catechin directly injected into animals is present and this is the first of its kind.

### Materials and Methods

Catechin was procured from Yucca enterprises, Mumbai. Soya lecithin, chloroform, methanol and all other chemicals used in this study were procured from Zeal chemicals and S.S Pharma, Hanamkonda and they were all of analytical grade. UV-Visible spectrophotometer was used for measurement of absorbances of different solutions. Heidolph flash rota evaporator, Bath sonicator, Magnetic stirrer (Remi Equipments Pvt. Ltd) were used for formulation of liposomes. A Waters HPLC system with a UV-Vis dual wavelength detector was used in this study. A Agilent C18 column was used in the HPLC analysis. Two PK softwares, WINNONLIN and KINETICA were used in the data analysis.

### Preparation and Characterization of Catechin Liposomes

Multilamellar vesicles encapsulating catechin were prepared by thin film hydration technique (Aukunuru et al., 2007). The formulation contained: catechin (25 mg), soya lecithin (60 mg) and normal saline (10 ml). Liposome preparation basically contained two steps: 1) formation of thin film: phospholipid i.e, soya lecithin, catechin were weighed as per the formula and dissolved in chloroform in a round bottomed flask of rota evaporator. 5 ml of ethanol was added to dissolve catechin. The solvent was then evaporated in a rotavapor under reduced pressure at a temp of 60 ± 5°C and flask was rotated at 90 rpm to create a thin layer of lipid film which was deposited on inner wall of round bottom flask. 2) Hydration of formed thin film: 10 ml of normal saline was added to the thin layer of round bottom flask to hydrate the layer. RB flask was rotated at 180 rpm at a temp of 70 ± 5°C for about an hour to form the suspension of liposomes. The liposomes obtained consist of multilamellar vesicles (MLVS). Small unilamellar vesicular liposomes were produced by sonicating MLVS in a bath sonicator. Sonication of MLV dispersion was accomplished by placing a test tube containing the liposome suspension in a bath sonicator and sonicated for 10-15 min above Tc of lipid. A transparent solution was obtained due to break down of MLVs to SUVs.

Particle size and *in vitro* drug release were used in the characterization of liposomes. An ordinary microscope was used for particle size measurement. Briefly, a drop of liposomal formulation was mounted on a slide and placed on a mechanical stage. The microscope eyepiece was fitted with micrometer by which the size of particles could be estimated. The field was focused on the slide and sizes of 50 particles were measured and the average was taken to determine the range of particles. Commercially available dialysis membrane was used which was placed in distilled water at 45°C for 30 min. The membrane was taken out and fixed in balloon shape to the tip of the test tube containing 1ml of liposomal formulation. The test tube was inverted and introduced into a beaker containing 50 ml of distilled water as dialysis medium. The beaker was placed on a magnetic stirrer to maintain vortex in the medium. Samples were withdrawn at intervals of one hour, for six hours, and then 24, 48, 72 hrs. up to 7 days. Medium was replaced with water each time when the sample was withdrawn. The samples were measured for absorbance using UV spectrophotometer at 280 nm.
HPLC Method to Determine Catechin Concentration in Plasma

Working initially with blank plasma spiked with standard catechins, several combinations of extraction media and mobile phases using a reverse-phase C18 column were investigated to find the optimum conditions. Extraction into methylene chloride followed by drying and reconstituting with the mobile phase gave the best recoveries. An aliquot of 100 μl of the plasma was taken in a microtube followed by the addition of 100 μl of methylene chloride because of which the protein in the sample was precipitated and this was removed by centrifugation. The methylene chloride remaining after modification was then injected onto the HPLC column. Prior to analysis it was filtered. HPLC method consisted of isocratic elution with methanol: water (adjusted to pH 2.5 with acetic acid): 20:80 as the mobile phase and the peak was detected at 280 nm. Retention time of catechin was 8.1 min and there was no protein peak in the chromatogram. The recovery for catechin was 62±5%. Standard calibration curves were linear over the range 2 ng to 2000 ng, with r.s.d. values of 6.2% and 7.2% for the inter- and intraday reproducibilities, respectively. The minimum detection limit was 0.2 ng where the noise to peak ratio was 1:3.

Animal Experiments

Nine healthy rats (3 months of age, weighing 200 gm) were used as experimental animals. During the experiment, the animals were maintained in individual cages and were fed and watered ad libitum. Animals were divided into three groups, each containing three rats. Male rats weighing 200-250 gm were selected. Group I received IV catechin solution. Group II and III received a single dose of catechin MLVs (IP) and catechin SUVs (IV), respectively. All the rats received 200 mg of drug in the form of solution or formulations. The IV bolus was administered into tail vein, and the IP injection at the ventral side near the stomach. Blood samples (0.5 ml) were collected from orbital sinus at 0, 0.5, 1, 2, 3, 6, 12, 24 hr after IV solution administration, and at 1, 2, 3, 6, 12, 24, 48, 72, 94, 120 hr after IP and IV administration of the liposomal formulations. Samples were collected into a tube containing an anticoagulant and after 10 min centrifuged at 1500 g for 15 min and plasma (0.25 ml) was collected and stored at -80°C (Jouan SA, Czech Republic) until analysis.

Data Analysis

All data in this study were presented as mean or mean±SEM. Pharmacokinetic parameters were determined using the two softwares Kinetica 4.4.1 and WinNonlin 1.1. Respective Graphs showing pharmacokinetics were also obtained using the same software. Data were analyzed by t-test using the statistical software MINITAB 14. Significance was recognized at P < 0.05.

Results and Discussion

The mean size of MLVs, as determined using an optical microscope was 1.2 ±0.1 μm. The particle size of SUVs was not determined as they were not visible under normal microscope. However, the particle size of SUVs is in nanorange and may be less than 1000 nm and could be conveniently injected into rat by IV route (Fukunaga et al., 1990). Percent loading in both the liposomal formulation was found to be around 80%. The drug release in vitro was sustained for 3 days Fig. 2A and 2B. Mean log plasma concentrations of catechin vs time data is shown in Fig. 3A, 3B and 3C after single IV bolus solution, single IV bolus formulation (SUV) and IP formulation (MLV) administration, respectively. The graphs were directly generated using WINNONLIN and the data was analyzed using the same and also with KINETICA. The best fit for IV bolus solution and SUV administration was achieved with a two-compartment i.v. input model, which was described by the mathematic equation:

\[ C = \frac{D(K_{21} - \alpha)}{(V_d(\beta - \alpha))}e^{\alpha t} + \frac{D(K_{21} - \beta) - (V_d(\beta - \alpha)).e^{\beta t}}{\alpha - \beta} \]

where, \( \alpha + \beta = K_{12} + K_{21} + K_{10} \), \( \alpha, \beta = K_{21}, K_{10} \), \( C \) = concentration, \( D \) = dose, \( V_d \) = apparent distribution volume, \( K_{21} = \) distribution rate constant from the peripheral compartment to the central compartment, \( K_{12} = \) distribution rate constant from central compartment to the peripheral compartment, \( K_{10} = \) rate constant associated with the elimination from the central compartment, \( \alpha = \) rate constant associated with the distribution phase of the concentration-time curve, \( \beta = \) rate constant associated with the terminal phase of the concentration-time profile and \( t = \) time. With IP MLV administration, the best fit was represented by a two-compartment open model with first order absorption and is a modification of previous equation with \( K_a \) as absorption rate constant. Pharmacokinetic variables with absorption phase were calculated using WINNONLIN and calculations were based on the equation described by Wagner (Aukunuru et al., 2002). As shown in Table 1, catechin has a half-life of 6.52 hr. The CL was 0.44 ml/h and the \( V_d \) was 10.85 L. After IV and IP administrations, the areas under the concentration time curves (AUC) was calculated using KINETICA. All the compartmental and noncompartmental PK parameters for liposomal formulations are shown in Table 1. The bioavailability of catechin administered with liposomal formulation compared to solution administration was determined from the ratio between AUC0-∞ IP pharmacokinetic variables were calculated for drug distribution after each administration.
Fig. 2  In Vitro Drug Release from Catechin Liposomes, A. MLVs B. SUVs.
Fig. 3 Plasma Log(Conc) vs Time Profile of Catechin After Several Administrations. (a). IV solution (b). IP MLVs (c). IV SUVs.

Table 1 Pharmacokinetic Variables of Catechin after single IP and IV Administration of MLVs and SUVs (800 mg/kg bw) (Mean±SEM)

<table>
<thead>
<tr>
<th>PK Parameter</th>
<th>IV administration</th>
<th>IP administration</th>
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<tbody>
<tr>
<td></td>
<td>Solution (n=3)</td>
<td>SUVs (n=3)</td>
</tr>
<tr>
<td>$C_{max}$ (ng/mL)</td>
<td>11.67±1.5*</td>
<td>19.46±2.5*</td>
</tr>
<tr>
<td>$T_{max}$ (hr)</td>
<td>0.5 ± 0.001*</td>
<td>1.00 ± 0.1*</td>
</tr>
<tr>
<td>$t_\frac{1}{2}$ (hour)</td>
<td>6.52 ± 1.01*</td>
<td>35.85 ± 2.32*</td>
</tr>
<tr>
<td>$K_{12}$ (h⁻¹)</td>
<td>1.05±0.11*</td>
<td>0.44±0.036*</td>
</tr>
<tr>
<td>$K_{21}$ (h⁻¹)</td>
<td>0.88±0.16*</td>
<td>0.302±0.026*</td>
</tr>
<tr>
<td>$K_{10}$ (h⁻¹)</td>
<td>0.1±0.021*</td>
<td>0.024 ± 0.0017*</td>
</tr>
<tr>
<td>$K_a$ (h⁻¹)</td>
<td>0.002±0.001</td>
<td></td>
</tr>
<tr>
<td>AUC total (ng.h/ml)</td>
<td>182.16±16*</td>
<td>863.74±24*</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>22.05±1.02*</td>
<td>197.05±2.19*</td>
</tr>
<tr>
<td>$V_d$ (L)</td>
<td>10.85±2.32*</td>
<td>92±2.64*</td>
</tr>
<tr>
<td>CL (mL/h)</td>
<td>0.44±0.05*</td>
<td>0.21±0.04*</td>
</tr>
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*P<0.05 (values with the same symbol are significantly different), $t_\frac{1}{2}$: the half–life, $K_a$: absorption rate constant, $K_{12}$: distribution rate constant for transferring the drug from the central to peripheral compartment, $K_{21}$: transfer from peripheral to central compartment, $K_{10}$: elimination rate constant, AUC: areas under the concentration time curves, MRT: mean residence time, $V_d$: volume of distribution, CL: total plasma clearance, $C_{max}$: maximal concentration in plasma, $t_{max}$: time to reach $C_{max}$
Hepatic fibrosis is a disease state characterized by exuberant synthesis and deposition of collagen in the extracellular matrix. Previously, the increased hepatoprotective effect of galactosylated liposomes encapsulated QC, another flavonoid like catechin, compared to its free form has been shown against NaAsO2 induced liver damage (Mandal et al., 2007) and this could explain the need and advantage of liposomal catechin in this pathology. Catechin is another promising antircirrhotic hepatoprotective agent. Its pharmacology is complex, with extensive metabolic conversions involved in the activation, inactivation and elimination of the drug. It is cleared via glucuronidation and biliary excretion (Feng, 2006). These drug properties also contribute to the marked heterogeneities in efficacy observed with catechin. Hence, drug carrier technologies represent a rational strategy to improve pharmacokinetics and biodistribution of catechin while protecting it from premature metabolism. We have used a liposomal strategy for this drug. Liposome-based systems have been used to enhance efficacy and/or ameliorate toxicity of certain drugs (Bonepally et al., 2007). Thus far, the most successful approach has involved constructs engineered for long circulation times, combined with stable encapsulation of the active compound within the liposome; this allows liposomes to accumulate at sites of the target, followed by rapid intracellular drug release. An example is PEGylated liposomal doxorubicin, which has received Food and Drug Administration approval for cancer treatment (Grenier et al., 2007). In this study, we investigated the pharmacokinetics of free and liposome encapsulated catechin. Two different liposomal formulations, one for sustained release and other for sustained release and targeting to specific liver cells were investigated. Catechin is an agent also useful in the treatment of several other diseases apart from cirrhosis. Thus, the results of this study also have a significant role where in catechin is otherwise useful. The results from this study indicate that for solution plasma-concentration time data fit best with a two-compartment open model. The drug has relatively moderate half-life and volume of distribution compared with other drugs is also lower suggesting good distribution into the body. As the possible cancer-preventive activity of tea is receiving a great deal of attention, information of PK of catechin definitely helps in understanding the biological effects of tea. Perhaps, this might the first report on pharmacokinetics of catechin after direct i.v. solution injection.

The data from IV catechin SUV injection was also best described by a two-compartment open model with, t1/2 and MRT of catechin from SUVs were higher, and CL was lower than catechin solution. The AUC value with SUV formulation was several fold higher than the solution form. In previous studies, on drug encapsulated in liposome, a similar pharmacokinetic findings were reported (Pardue and White, 1997). These results may depend on sustained release of drug, and after initial uptake of drug-loaded liposomes from the blood by phagocytic cells, the RES may act as a reservoir of drug, releasing it slowly back to the body (Ellens et al., 1982). In this study, it was determined that the Vd with SUVs was several times higher than solution. In fact, SUVs had a large Vd, which indicated that there was good penetration of the drug into a wide range of tissues and it could have a longer therapeutic effect duration than solution form. In a previous study, similar result with ampicillin formulations was achieved with Vd of liposome encapsulated ampicillin was higher than that of the free drug in sheep (Pardue and White, 1997).

The data obtained from IP administration of MLVs was best represented by a two compartment open model with absorption parameter incorporated, the parameters were determined using WINNONLIN and the model employed is shown in Fig. 4. After IP administration of MLVs, drug concentration reached Cmax within a longer time (P<0.05) and t1/2 was higher than those of catechin solutions. The results are similar to studies performed with other drugs, administration routes and species (Elmas et al., 2002). The results suggest that the absorption of catechin in liposomes from injection sites was slower. After parenteral administration, the sustained mechanism of release from liposomes after other than IV route such as IM, SC and IP, is not clearly explained. However, some theories have been postulated. It was previously (Ohsawa et al., 1985) speculated that liposomal structure can be destabilised because of various factors, and the drug is absorbed in a free form from the place of administration. The other suggestion was that liposomes injected by the IP route entered into the lymphatics and only drug released from liposomes diffused directly into the systemic circulation. Similarly, another study described that once injected, liposomes remain in the structure of tissue forming a compressed depot from which the drug is released, slowly. In this study, it was determined that the Cmax of catechin with MLV was lower than that obtained out of injecting solution. After IP administration of liposome encapsulated drugs, which acted as a local depot, there was a slower release, lower Cmax and long-lasting concentrations of active agent in the plasma compared with administration of the free form. After IP administration of catechin formulation, the low Cmax and plasma concentrations may cause a reduction in dose-dependent side effects of the drug (Eichler et al., 1985). In the present study, t1/2 and MRT of catechin from MLVs were longer and CI was lower than those of catechin obtained out of injecting a
solution. Absorption is the process that determines prolonged plasma concentration and consequently the elimination half-life increase (flip-flop phenomena). These results suggest that liposome encapsulated drug formulations provide longer effective concentrations in plasma. In this study, \( V_d \) of catechin after IP administration of MLVs was higher and this indicates better penetration of the drug into a wider range of tissue than in the case of injecting a solution.

**Fig. 4** Pharmacokinetic Models Used in This Study A. IV Bolus Solution and IV Bolus SUV Administration. B. IP MLV Administration.

Liposomal catechin in this study is composed of soyalecithin only, and do not include either cholesterol nor it is pegylated. Liposomal encapsulation prolongs exposure to doxorubicin, although pegylation results in longer exposure than non-pegylated liposomal catechin. Incorporation of cholesterol and pegylation of catechin liposomes is yet to be investigated. Thus, multilamellar liposomes vesicles used in this study which are widely used as carriers for sustained release of the drug can sustain the release of catechin in the plasma. On the other hand, SUVs are preferentially trapped by phagocytic cells in KC and LSEC in liver. Prolonged circulation time may increase the therapeutic index of catechin entrapped in liposomes against cirrhosis. The formulations developed by our group for the treatment of cirrhosis can also be for other diseases where in catechin might be of potential help. For instance, catechin molecules encapsulated in liposomes can extravasate into tumours with abnormal vascular endothelium but may not penetrate normal tissues thereby making such a formulation useful for targeting the drugs.

**Conclusion**

In conclusion, when liposomal formulations are compared to solution, formulation demonstrated a lower \( C_{max} \), longer \( t_{1/2} \), higher MRT, larger \( V_d \) and slower Cl, and provides effective and prolonged plasma concentration in the body after IV and IP administration. In addition, when catechin was administered in a liposome entrapment form it had long duration of activity threshold, this result may be beneficial for the diseases in which catechin is useful.

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