Preparation, evaluation and pharmacokinetics of diosmin herbosome in beagle dogs

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Abstract: Diosmin is one of the most widely used phlebotonic drugs, but its poor bioavailability has restricted its usage. The aim of this study was to formulate a complex Diosmin with phospholipids (75% in PC, in 1:2 molar ratios) and to evaluate for solubility, drug content, X-ray diffraction (XRD), differential scanning calorimetry (DSC) and in vitro dissolution study. Further to test the bioavailability of both the complex and Alvenor® in beagle dogs and compare pharmacokinetic parameters. Diosmin herbosome was found to be more soluble than both pure diosmin and Alvenor®. The complex contained 71.94% drug content. DSC thermograms and XRD also proved the claim of the complexation. The dissolution profile of diosmin herbosome and Alvenor® in water-ethanol medium showed an increase of the dissolution for diosmin herbosome. Comparison of plasma concentration and main pharmacokinetic parameters of diosmin herbosome treated and Alvenor® treated dogs showed a higher Cmax for the complex with longer elimination half-life. The complexation of diosmin with phospholipids can be potentially used in enhancing the absorption and solubility, consequently increasing the bioavailability of the drug.

Keywords: Bioavailability, enhancement, diosmin, herbosome, dogs.

INTRODUCTION

There is an extensive body of literature examining therapeutic activities of diosmin (Bedada and Boga, 2017; Bogucka-Kocka et al., 2013; Filho et al., 2018; Queenthy et al., 2018). One of its main actions is the treatment of venous diseases (Hnatek, 2015). It is also used in the management of hemorrhoids (Giannini et al., 2015) and to reduce swelling after surgeries. Diosmin has an effect against heart problems and blood pressure (Queenthy and John, 2013; Senthambizhvelan et al., 2014), diabetes (Jain et al., 2014), along with several types of cancer (Lewinska et al., 2015; Naso et al., 2016). In addition, it has potential anti-inflammatory (Imam et al., 2015) and antioxidant (Naso et al., 2016) actions. Despite this demonstrated efficacy, its poor solubility along with poor oral bioavailability compromise the potential for therapeutic uses. Diosmin is completely degraded by the intestinal bacteria into its aglycom diosmetin (Silvestro et al., 2013), which is systemically absorbed (Walle, 2004). Diosmetin is then rapidly glucuronic-conjugated in the rat and provides an explanation for the low bioavailability of diosmin.

In order to fully utilize the potential of this agent, it is necessary to improve its bioavailability. Therefore, phospholipids are used to enhance the bioavailability of some drugs by modifying their rate of release, improving their solubility and facilitating their permeation (Amit et al., 2013). The phospholipids complex increases the absorption of active ingredients when topically applied on the skin (Tsai et al., 2015) and improves systemic bioavailability when orally administered. Pharmacokinetic studies in different animals like rats and dogs as well as in humans have shown increased bioavailability (Gupta and Dixit, 2011; Semalty et al., 2012). In a comparative study, the curcumin absorption was higher for Meriva® as compared to the unformulated curcumin (Zhang et al., 2013). At the same dose, the action of the 18β-glycyrrhetinic acid phytosomes was found to be greater and to last longer than 18β-glycyrrhetic acid alone. Similar is the case with many others such like; ginkgoselect phytosome and green select phytosome (Maryana et al., 2016). This means that the phospholipids complex does not only increases the active ingredient tolerability and absorption but also improves its efficacy (Mishra et al., 2012).

In this study, we seek to prepare a complex of diosmin with phospholipids to overcome its poor bioavailability along with enhancing its efficiency under low doses.

MATERIALS AND METHODS

Biological and chemical materials
The materials included; raw diosmin, soya phosphatidylcholine (PC), commercially available diosmin (Alvenor®) dichloromethane, methanol, dioxane, n-octanol, microcrystalline cellulose, magnesium stearate, talc, silicon dioxide, emcosoy, croscarmellose sodium and cornstarch.

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Preparation of diosmin-pc complex and physical mixture
To optimize the preparation technique in terms of solubility and drug content, different kinds of phospholipids (PHOSPHOLIPON® 50, LIPOID® S100, PHOSPHOLIPON® 85G and LIPOID® S75) were tested. The complex was tried for two different molar ratios (1:1 and 1:2) of the drug to phospholipids. In addition, other conditions such as the reaction time and temperature were also investigated.

Finally, the complex was prepared using LIPOID® S 75 in 1:2 molar ratios as it was found optimum for complex formation. The PC was first dissolved in 10 ml of methanol and then was added to 90 ml of diosmin dissolved in dioxane. This mixture was first refluxed for 2 h then, evaporated to small volume under vacuum at 40°C in a rotary vacuum evaporator (Shanghai Biochemical Equipment®, R-202). The residue was re-dissolved in dichloromethane and was stirred for 1 h. The solvent was evaporated afterward, the collected residues were invested in the vacuum desiccators overnight and then were characterized.

Physical mixtures were prepared manually by mixing the appropriate quantities of diosmin and phospholipids in a mortar for 30 min.

Preparation of dosage forms
Due to waxy nature of the prepared complex, its formulation to oral dosage forms possibly faced a lot of obstacles. A direct volumetric filling process can be applied, even if the apparently low density of the herbosome seems to limit the maximum amount of powder that can be filled into a capsule. To modify the stickiness of the final complex and monitor the release time, many excipients were tested.

Pre-formulation Studies
Drug content and entrapment efficiency
A quantity of complex was dissolved in an appropriate volume of water. The solution was subjected to ultracentrifugation (15800r/min for 20 min). The supernatant and pellet were separated in two different tubes. Dilutions were made suitably and measured at 254 nm using UV-2401 PC UV/Visible recording spectrophotometer (Shimadzu, Japan). The test was prepared in triplicate.

Solubility
Alvenor®, physical mixture and pure diosmin, complex were dissolved, both in water and n-octanol, in order to determine any variation in the solubility of these compounds due to complex formation. An excess of diosmin was added to 5 ml of each solvent. The solutions were put on continuous stirring for 24 h, thereafter, centrifuged for 20 min. The suitable delusions were made and the drug content was measured at 254 nm. The test was prepared in triplicate.

In vitro dissolution study
In vitro dissolution studies for herbosome capsules and Alvenor® were performed in triplicate using a ZRS-8G (Bio-Equip®) six station dissolution test apparatus. The paddle method was set at 120 rpm, 37°C and 1000 ml of water/ethanol mixture (9:1). The dissolution fluid was sampled at different intervals and an equal amount of new media was added. These samples were then filtered and their suitable dilutions were made which were then studied at 254 nm.

Evaluation and characterization
Differential Scanning Calorimetry Analysis
Thermograms of pure diosmin, PC, herbosome and the physical mixture were recorded using a differential scanning calorimeter (NETZSCH®, DSC 204). Each sample was covered under the pan, having a uniform gas nitrogen flow for its thermal behaviour studies. At a constant rate of 10°C per minute, the samples were heated from the range of temperature, 25-300°C, with gradual heating.

X-ray powder diffraction analysis
The crystalline state of pure diosmin, PC, herbosome and the physical mixture were evaluated with XRPD. D8 Advance Powder X-ray diffractometer (Bruker®) was employed to obtain the diffraction patterns.

The X-ray generator that receives a tube voltage of 40 KV, tube current of 40 MA and Ka lines of copper was employed to generate the radiations. Meanwhile, the angle was set in step scan mode at 2 θ ranged at 3-40°C of temperature.

Bioavailability study and pharmacokinetics in beagle dogs
The enhanced bioavailability potential of the herbosome was tested in eight beagles (8.6-10.7 kg), randomly placed into two groups. Each group received either herbosome capsules or Alvenor® tablets, having equivalent quantities of diosmin (450 mg). After a period of washout, the groups were switched; this enabled the data collection on the same set of dogs. Two milliliters of blood was collected in heparin tubes after 30 min, 1, 2, 4, 5, 6, 7, 8, 10, 12, 24, 36 and 48 h of oral administration. After centrifugation, plasma samples were collected and kept at -20°C. The guidelines for health and care for the experimentation on animals were followed in true spirits as recommended by the National Institutes of Health.

Sample preparation and lc-ms-ms analysis
First, a mixture of 125 μl of NaOAc (1 M, pH 5.0) and 0.4 ml of thawed plasma was incubated with 200 μl of B-gluconidase type H-1 (Sigma-Aldrich, St. Louis, MO,
USA) in a constant temperature shaker SHA-B (China) at 37°C and 100r/min for 18h. Then, 40μl of internal standard (3-Methylflavone-8-carboxylic acid), 1ml of NaCl (0.9%), and 200μl of HCl (1 M) were successively added and the mixture was agitated for 3s. Finally, 4 ml methyl tetra butyl ether/isopropanol mixture (1:1) was put and the mixture was again stirred again for 3 min. After thorough centrifugation, the mixture was heated to evaporate the organic phase under vacuum at 37°C. The resultant mixtures were again re-formed in 150μl of mobile phase and were again centrifuged (10min, 4000 rpm).

Samples of 20μl were analyzed using a Phenomenex Gemini C18 column (150 × 4.6 mm, Phenomenex®, Torrance, CA, USA) on a Finnigan TSQ Quantum Ultra AM triple-quadruple tandem mass spectrometer (Thermo Electron Corporation®, San Jose, CA, USA) having a source of electrospray ionization in its negative mode that is monitored at a wavelength of 288 nm. A mixture of acetonitrile: water-0.1% formic acid (55:45) was used to separate the mixture a 5min. Pure diosmin and its dilutions, prepared in a solution mixture of methanol and DMSO, were subjected to generate the standard curves. The spikes were observed on standard curves with internal standard. The same were prepared for the extracted and reconstituted for plasma samples and studied. For the sake of acquisition of the data and instrument control, Xcalibur™ workstation software (Version 1.4 SR1, 2003, Thermo Scientific™) was employed. The validation of the technique used was done by the addition of know different amounts of diosmin to the blank plasma of the dogs. The resulting concentrations of diosmin were 0.1, 0.2, 0.5, 1.0, 2, 5, 10, 15, 30 and 40 ng ml⁻¹. In order to test the precision and accuracy as well as linearity of the technique, analytical process was conducted on the dilutions.

**STATISTICAL ANALYSIS**

The data obtained was analyzed by Analysis of variance (ANOVA) technique using SPSS version 22 statistical computer software at 5% probability.

**RESULTS**

*Preparation of the complex and dosage forms*

Results have shown that the molar ratio and time of reaction played an important influence. The temperature has no or small effect on the quality of the prepared complex. Phospholipon 50 has been shown to be the best-used phospholipid giving high solubility and high drug content but it has been avoided in our preparation due to the big quantity needed to prepare 1:2 diosmin phospholipids complex because phospholipon 50 contains just 45% of PC which makes its preparation to dosage forms impossible. Finally, the best herbosome was prepared using lipoid S75 in the molar ratio 1:2 at 40°C refluxed first in a mixture of dioxane/methanol (7/3) for 2 h and then in dichloromethane for 1 h.

To prepare a tablet of the complex with a dose of 450 mg in diosmin, 70 % of excipients were used to minimize the stickiness of the complex and made it suitable for compression. On the other hand, direct compression is avoided due to the high unitary dose. The characteristics of the complex make its formulation into tablet impossible. To overcome this problem, an attempt was made to prepare hard gelatine capsules with just little quantity of excipients. To optimize the characteristics of the complex, many batches were prepared using different excipients in different concentrations. The results showed that the batch prepared using 1% croscarmellose, 10% MCC, 2% silicon dioxide and 1% magnesium stearate showed a quick disintegration within just 1 min.

*Differential scanning calorimetry*

The results of the DSC test confirmed the association of diosmin and PC in the complex as both peaks representing diosmin and PC changed position (fig. 1). Thermal analysis of the drug alone demonstrated characteristic thermograms with three endothermic peaks at 51.9°C, 101.1°C and 275.4°C. The thermograms of diosmin-PC complex showed a disappearance of the diosmin peak and appearance of new endothermic peaks at low onset temperatures. There was also change in the shape of the peak.

*X-ray powder diffraction*

In order to assess the possible variations in the morphology of the diosmin crystal pertaining to the polymorphic transition and to investigate the diosmin-PC complex in the solid state, we carried out XRPD analysis. fig. 2 shows the powder XRD patterns of pure diosmin, PC, and their complex. The diffraction pattern of diosmin powder diffraction exhibited a fractional sharp crystalline peaks, which is a peculiar feature of a molecules that has undergone crystallinity.

**Fig. 3**: Dissolution profile of diosmin-phospholipds phytosome capsules and micronized diosmin tablets

*Drug content and solubility*

The complex showed the same absorption spectra as pure diosmin confirming the theory of physical complexation. This complexation did not lead to the formation of a new
Table 1: Solubility comparison among various preparations (n=3).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Solubility in aqueous layer (µg/ mL)</th>
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<tr>
<td>Pure diosmin</td>
<td>1.82 ±0.16</td>
</tr>
<tr>
<td>Alvenor®</td>
<td>3.92 ±0.04</td>
</tr>
<tr>
<td>Diosmin tablet</td>
<td>2.63 ±0.05</td>
</tr>
<tr>
<td>Diosmin-phospholipids complex</td>
<td>18.29 ±0.13</td>
</tr>
<tr>
<td>Physical mixture</td>
<td>10.54 ±0.06</td>
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Data expressed as mean values and standard deviations (± SD); n=3

Table 2: The pharmacokinetic parameters of diosmin complex and Alvenor® following a single dose of 450 mg (mean±SD, n = 8).

<table>
<thead>
<tr>
<th>Dosage</th>
<th>Diosmin Complex</th>
<th>Alvenor®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (ng/mL)</td>
<td>0.71±0.62</td>
<td>0.47±0.44</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>4.5±5.2</td>
<td>13.3±15.7</td>
</tr>
<tr>
<td>MRT0-72 (h)</td>
<td>86.57±82.40</td>
<td>67.66±28.89</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>55.24±60.21</td>
<td>39.69±20.22</td>
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Fig. 1: DSC thermograms of Pure diosmin, Lipoid s75, Physical mixture and Diosmin-phospholipid complex.
Fig. 2: X-Ray Powder Diffraction (XRPD) study of (a) Diosmin (b) PC (c) Diosmin complex (d) Physical mixture
compound. Diosmin is just enrolled by phospholipids through non-covalent bonds. The content of diosmin in the complex was 71.94% (w/w). Herbosomes showed a high percentage of drug loading that makes its delivery clinically feasible.

**DISCUSSION**

The present study was undertaken to study the formulation of Diosmin phospholipids preparation and study the pharmacokinetics of this complex in the Beagle dogs and in vitro dissolution behavior. The results were compared with Alvenor®. In this study, the endothermic peak observed at 275.4°C by differential scanning calorimetry is attributed to the melting point of diosmin, however, Freag *et al.*, (2013) recorded this endothermic peak at 291.5°C. The degree of purity of the diosmin seems to be the reason behind the variability in the melting points. Pure phospholipids exhibited endothermic peaks at 43.4°C, 175.8°C, 215.4°C and 242.0°C. The first mild peak is probably due to the slightly hot raise in temperature that lead to the vigorous movements of polar heads of PC. Similar mild peak was also reported by Semalty *et al.* (2012). The second and third peaks represent the melting of PC in two phases during which transition of phase occurred from gel to liquid crystalline. Gupta and Dixit (2010) recorded two peaks at 113.29°C and 185.37°C of phosphatidyl choline. Freag *et al.* (2013) observed a single peak at 165.4°C. The difference of this may be attributed to different type of phospholipids used in both studies. Further, it was found that the physical mixtures reflected endothermic peaks at 125.1°C and 183.3°C and an exothermic peak at 240.7°C. There is also a difference with the experiments of Freag *et al.* (2013) that may be attributed to the percentage purity of diosmin, type of PC and their resulting complex.

The changes in the thermograms of diosmin-PC complex are attributed to some interactions like hydrogen bonding or van der Waals forces or combination of both may exist between diosmin and phospholipids as reported by Xu *et al.* (2009). After combining the dosmin and PC, the hydrocarbon chain of phospholipid could rotate freely and envelops the polarity parts of phospholipid molecules that made the sequence, reduced between aliphatic hydrocarbon chain of phospholipids exhibited endothermic peaks at 43.4°C, 175.8°C, 215.4°C and 242.0°C. The first mild peak is probably due to the slightly hot raise in temperature that lead to the vigorous movements of polar heads of PC. Gupta and Dixit (2010) recorded two peaks at 113.29°C and 185.37°C of phosphatidyl choline. Freag *et al.* (2013) observed a single peak at 165.4°C. The difference of this may be attributed to different type of phospholipids used in both studies. Further, it was found that the physical mixtures reflected endothermic peaks at 125.1°C and 183.3°C and an exothermic peak at 240.7°C. There is also a difference with the experiments of Freag *et al.* (2013) that may be attributed to the percentage purity of diosmin, type of PC and their resulting complex.

The XRDP of diosmin-PC complex revealed peaks similar to PC indicating that the diosmin in phospholipids complex was either molecularly dispersed or in amorphous form (Semalty *et al.*, 2012). The formation of the complex is confirmed from the disappearance of the diffractional peaks of diosmin. The few remaining peaks were due to free diosmin. Our findings supports the
previous reports by Cui et al., (2006); Semalty et al., (2009a; 2010b; 2012); Shi et al., (2006) and Yoo and Park (2003).

Regarding the solubility, recording of vesicle formation in the media and amorphous nature of the complex explains the phenomenon of increased solubilization. The wetting and dispersion action of the phospholipids and their amphiphilic surfactant characteristics, the solubility of the drug could be increased. Gupta and Dixit (2011) explained this phenomenon indicating the amorphous nature of the complex.

From the bioavailability results, it is quite evident that phospholipids do not metabolize as fast as Alvenor® does. Alvenor® does not stay as long as the complex. This is why complex shows a notably higher bioavailability. These data show that a herbosome complex of PC and diosmin markedly enhances bioavailability in dogs. This is because of increased membrane permeability of the complex as membrane is a bilayered lipid moiety which facilitates the lipid particles across it and hence better biological effects (Semalty et al., 2009 and 2010).

CONCLUSION

In the present study, diosmin herbosome (1:2 molar ratio) was successfully prepared using a simple solvent evaporation method. DSC and XRD curves showed that drug and phospholipids combined and formed a complex with higher solubility and better dissolution profile compared to the physical mixture, pure diosmin and also Alvenor®. Studies on beagle dogs showed an increase of Cmax with sustained release and longer time of elimination. The complexation of diosmin with soybean phospholipids increased its solubility, thus has remarkably enhanced its bioavailability.

REFERENCES


