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Pharmacokinetic Profile of µSMIN Plus™, a new Micronized Diosmin Formulation, after Oral Administration in Rats

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Diosmin is a naturally occurring flavonoid present in citrus fruits and other plants belonging to the Rutaceae family. It is used for the treatment of chronic venous insufficiency (CVI) for its pheblotonic and vaso-active properties, safety and tolerability as well. The aim of the current *in vivo* study was to investigate the pharmacokinetic profile of a branded micronized diosmin (µSMIN Plus™) compared with plain micronized diosmin in male Sprague-Dawley rats. After oral administration by gastric gavage, blood samples were collected via jugular vein catheters at regular time intervals from baseline up to 24 hours. Plasma concentrations were assessed by LC/MS. For each animal, the following pharmacokinetic parameters were calculated using a non-compartmental analysis: maximum plasma drug concentration (C_{max}), time to reach C_{max} (T_{max}), area under the plasma concentration-time curve ($AUC_{0\text{-}last}$), elimination half-life ($t_{1/2}$), and relative oral bioavailability (%F). The results of the current study clearly showed an improvement in the pharmacokinetic parameters in animals treated with µSMIN Plus™ compared with animals treated with micronized diosmin. In particular, µSMIN Plus™ showed a 4-fold increased bioavailability compared with micronized diosmin. In conclusion, the results from the current study provided a preliminary pharmacokinetic profile for µSMIN Plus™, which may represent a new tool for CVI management.

Keywords: µSMIN Plus™, Dosmin, Nutraceuticals, Pharmacokinetics, Chronic venous insufficiency.

Diosmin (3',5',7'-trihydroxy-4'-methoxyflavone-7-rhamnogluco-side) is a naturally occurring flavonoid present in citrus fruits and other plants belonging to the Rutaceae family. Diosmin is constituted by a sugar moiety (rutinoside disaccharides) linked to the aglycone diosmetin; it could be obtained by extracting hesperidin from citrus rinds, through iodine-assisted oxidation.

Diosmin was isolated in 1925 and has been used as a therapeutic agent for 40 years. Nowadays, it is used as a vascular-protecting compound for the treatment of chronic venous insufficiency (CVI), hemorrhoids, and varicose veins for its pheblotonic and vaso-active properties, and for its safety and tolerability as well [1]. Following oral administration, diosmin is rapidly hydrolyzed by enzymes of enterobacteria into the aglycone, diosmetin, which is then absorbed through the intestinal mucosa [2]. Experimental evidence showed that glucuronidation occurs rapidly in rats [3] and diosmetin circulates as a glucuro-conjugated compound in blood.

The sugar moiety is a major determinant for the absorption and bioavailability of flavonoids and deglycosylation represents the first and crucial step for their metabolism and absorption.

For poorly soluble compounds, the intestinal absorption is influenced by different factors including dissolution rate. Several attempts have been carried out to enhance the bioavailability of such flavonoids for example removal of rhamnose from the rutinose moiety of hesperidin, a flavonoid structurally related to diosmin, commonly found in *Citrus sinensis* fruits. Following oral administration in healthy volunteers, the aglycone was highly bioavailable [4].

Micronization successfully enhanced the bioavailability of poorly water-soluble compounds such as diosmin, hesperidin, and quercetin [5]. The common method to obtain micronized particles is by mechanical pulverization of larger particles (jet milling, ball milling, pin milling); the lowest particle size that can be achieved is about 1–3 µm. Particle size reduction is widely used to increase dissolution rate and, consequently, the surface of intestinal epithelium in contact with compounds is increased and this contributes to the improvement in absorption. The aim of the current study was to assess the pharmacokinetic profile of a branded micronized diosmin (µSMIN Plus™) compared with plain micronized diosmin in male Sprague-Dawley rats after oral administration.

Liquid chromatography including a Gemini C18 column with gradient elution using a water and methanol mobile phase showed a good separation of analytes. Four analytical sessions were carried out and all were accepted. The linear calibration curves in the examined concentration range of 0.05 to 1.0 µg/mL showed a $R^2 \geq 0.996$, and precision and accuracy values within the tolerated limits (Table 1) with the limit of quantitation represented by the lowest point on the calibration curve.

Table 1: inearity, precision, and accuracy of the method for the analysis of diosmetin in rat plasma samples over 4 analytical sessions. SD: standard deviations.

Theoretical concentrations (µg/mL)	Mean concentrations (µg/mL)	SD	Precision (%)	Accuracy (%)
0.050	0.049	0.007	14.58	-1.50
0.100	0.097	0.008	7.92	-2.50
0.250	0.246	0.004	1.64	-1.73
0.500	0.509	0.001	2.50	1.80
1.000	1.027	0.066	6.43	2.72

The inter-assay precision and accuracy were evaluated from repeated analysis of quality control samples. The precision was less than 9.28% and accuracy within -4.6 – 4.2 % (data not shown).

The disposition of diosmetin was investigated following a single oral dose (50 mg/kg) of two different formulations of diosmin and

evaluating concentrations of diosmetin in plasma samples collected at different times after dosing. After administration of diosmin formulations by oral gavage, no presence of this flavonoid and its aglycone, diosmetin, was found in rat plasma. With both formulations, diosmetin concentrations (expressed as aglycone) were measured in rat plasma after enzymatic hydrolysis with liver bovine β-glucuronidase.

Plasma concentrations of diosmetin are shown in Figure 1. After plain micronized diosmin treatment, plasma diosmetin concentrations were below the limit of quantification. However, a 2-fold increase of diosmetin concentration at 6-8 h post dosing was found compared with initial plasma appearance of the compound at 0.25 h. Due to a poor concentration-time profile, only the AUC_{last} pharmacokinetic parameter was enabled to be evaluated (0.79 ± 0.36; µg/h/mL) (Table 2).

Table 2: Pharmacokinetic parameters of diosmetin following oral administration of micronized diosmin and µSMIN Plus™ (50 mg/kg b.w.) in rats.

Parameters	Micronized diosmin	µSMIN Plus™
K_{el} (h^{-1})	Not applicable	0.09 ± 0.02
$T_{½}$ (h)	Not applicable	7.70 ± 1.49
C_{max}(µg/mL)	0.046 ± 0.01	0.31 ± 0.07
T_{max} (h)	8.00 ± 2.83	3.25 ± 1.67
AUC_{last}(µg/h/mL)	0.79 ± 0.36	3.15 ± 0.16
Relative bioavailability (F)		3.98

Following µSMIN™ Plus administration, plasma diosmetin concentrations rose slowly and reached a maximum concentration (C_{max}) of 0.31 ± 0.07 µg/mL within 3.25 ± 1.67 h (T_{max}), then declined with detectable values (C_{last} = 0.04 ± 0.01 µg/mL) at 24 h, with an apparent $t_{½}$ of 7.699 ± 1.49 h (Table 2). The relative oral bioavailability (F) of diosmetin between µSMIN™ Plus and normal micronized diosmin, calculated from the ratio of AUCs of diosmetin after each treatment, (3.15 ± 0.16 µg/h/mL and 0.79 ± 0.36; µg/h/mL, respectively) was 3.98 (Table 2).

The LC-MS method, developed for the quantitation of diosmetin in plasma of rats orally administered with two different formulations of diosmin (50 mg/kg b.w.) showed no presence of diosmin and unconjugated diosmetin in plasma of treated animals in the range of the examined concentrations (0.05-1.0 µg/mL and 0.5-10 µg/mL for diosmetin and diosmin, respectively). To our knowledge, no literature data about diosmin oral administration in rats are available. However, when diosmetin was orally administered as its aglycone to rats, high levels of conjugated glucuronides were found in both blood and urine [3]. This finding suggests that diosmetin was orally available and circulates in the rat body in its conjugated form. Similar results were found during oral administration of micronized diosmin in healthy volunteers [6, 7].

All of these clinical studies have shown that diosmin is rapidly hydrolyzed by the enzymes of intestinal bacteria into diosmetin, which is subsequently absorbed and further metabolized to conjugated diosmetin. However, the presence of diosmetin in human plasma remains questionable, as shown by Cova *et al*. [8] and Kanaze*et al.*[9]. A possible explanation of this discrepancy could be the different analytical methods used in the clinical studies.

This issue is crucial to address on the active pharmacological moieties of diosmetin, as the major circulating metabolites of diosmin are diosmetin glucuronides [3, 6, 7]. This finding is supported by our experimental investigation following oral administration of diosmin (50 mg/kg b.w.) in rats. Thus conjugated diosmetin may persist in the circulation for long periods and

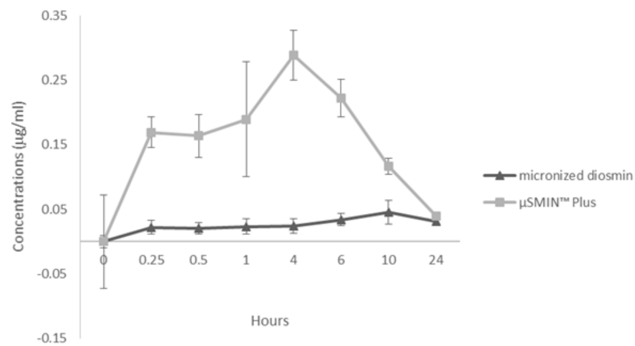

Figure 1:Pharmacokinetic profile of diosmetin following oral administration of micronized diosmin and µSMIN Plus™ (50 mg/kg b.w.) in rats. Plasma concentrations (µg/mL) are shown as mean values ± standard deviations (n. = 5 animals/group).

consequently their bioactivity may have pharmacologically significant effects. However, the mechanisms of absorption (e.g. active or passive transport) and first-pass effect (e.g. gut or liver location) of diosmin and/or diosmetin remain to be adequately established in humans and appropriate animal models.

An interesting finding of the current study was the high plasma concentration of diosmetin following oral administration of µSMIN Plus™ compared with plain micronized diosmin. This is indicative of a better absorption of the compound from the rat gut compared with conventional micronized diosmin. From a pharmacokinetic point of view, a good formulation of diosmin with excellent absorption could produce less pharmacokinetic variation in subjects undergoing diosmin treatment. Furthermore, an increase (~400%) of relative bioavailability of µSMIN Plus™ compared with micronized diosmin could imply a reduction of the administered dose needed for reaching similar pharmacologic effects.

In conclusion, the findings of the current study showed a better pharmacokinetic profile of µSMIN Plus™ in comparison with conventional micronized diosmin. Finally, we can assume that µSMIN Plus™ might be a useful ingredient for dietary supplements intended for supporting symptomatic treatment of chronic venous insufficiency (CVI) and, in general, for improvement of microcirculation and vascular tropism showing an enhanced pharmacokinetic profile compared with micronized diosmin. Further studies are desirable to understand better the mechanism of action and distribution of diosmin in humans.

Experimental

Materials: µSMIN Plus™ and micronized diosmin were supplied by Giellepi S.p.A. (Milan, Italy). Analytical standards (diosmin, diosmetin, 7-hydroxy-trifluoro-methylcoumarin), all reagents and LC/MS grade solvents (acetonitrile, methanol, acetic acid) and bovine β-glucuronidase (from bovine liver, Type B-1) were purchased from Sigma-Aldrich (Milan, Italy). Ultra-high quality water (water Optima LC/MS, Fisher Chemical, USA) was used for chromatography.

Animals: Sprague-Dawley rats (Charles River srl, Calco, Italy), 150-175 g body weight, 6-8 weeks of age, were housed in rooms having controlled environment conditions. Throughout the experiment, which was performed after a minimum of 7 days after arrival of the animals, drinking water was supplied *ad libitum*. Each rat was offered daily a complete pellet diet (4RF21 GLP, Mucedolasrl, Milan, Itlay) throughout the study. Physical appearance, behavior and general clinical signs of rats were observed throughout the experiment. Any deviation from normality was recorded.

Treatment: Ten male rats (5 rats/group according to treatment) were orally administered by gavage with either micronized diosmin or µSMINPlus^{TM} (50 mg/kg b.w.). Blood samples were collected from a jugular vein catheter at 0.25, 0.5, 1, 4, 6, 10 and 24 h and centrifuged immediately at 8000 rpm for 5 min. The resultant plasma was stored at -20°C until analysis. Concentrations of diosmetin were evaluated by a LC/MS method.

Procedures: All the animals were weighed before either jugular vein catheterization or administration of the test item compound. For catheterization, rats were anesthetized with isoflurane (2-3%) and a ~18 cm polyurethane tubing (PTPU-040 ID=0.63mm; OD=1.02 mm) implanted in the right jugular vein for blood sampling. The tube crossed the subcutaneous tissue and was fixed at its exit from the dorsal neck region at a port device. Tubes filled with heparinized saline solution (25 U.I./mL) were used in order to avoid occlusions due to blood clots. After surgery, rats were housed individually in plastic cages and allowed to recuperate for one day before the treatment. The gavages were conducted with a 2.5 mL syringe (Chemils.r.l., Padova, Italy) equipped with ball-tipped stainless steel gavage needles. The animals were fasted 12 h before the treatment with both formulations.

After administration of diosmin formulations, 0.20 mL of blood was collected via jugular vein catheters with zero-dead volume disposable syringes (1 mL iv insulin syringes with attached 25G 5/8 inch needles; Chemils.r.l., Padova, Italy) at 0.25, 0.5, 1, 4, 6, 10 and 24 h post dosing. After each blood withdrawal, the catheters were washed with 0.15 – 0.2 mL of sterile physiological saline and filled with 0.03 mL of heparin saline solution (0.25 U.I./mL). The heparinized blood was immediately centrifuged (8000 rpm for 5 min at 4°C) and the resultant plasma transferred into vials and stored at -20°C until analysis.

Sample analysis: The concentrations of diosmetin in plasma were evaluated by ultra high performance liquid chromatography-high resolution mass spectrometry (UHPLC/HRMS). To measure diosmetin in plasma, a deconjugation with β-glucuronidase was performed in order to liberate diosmetin and quantify it. Enzymatic hydrolysis to produce diosmetin was achieved by adding to 50 µL of plasma, 20 µL of β-glucuronidase (10000 U.I./mL in water) and 125 µL of acetate buffer (200 mM, pH = 4.6). The sample was incubated at 37°C for 24h with constant mixing at 800 rpm/min (Thermomixer comfort, Eppendorf, Hamburg, Germany). In order to terminate the enzymatic reaction, 300 µL of CH_3CN containing IS (7-hydroxy-trifluoro-methylcoumarin; 15 ng/mL in CH_3CN) were added to a 2.0 mL Eppendorf polypropylene tube. The mixture was vortex-mixed for 1 min, kept either in ice or at -20°C for 20 min, vortex-mixed for 1 min, sonicated for 1 min and centrifuged at 14000 x *g* for 10 min at nominally 4°C. The supernatant aliquot (~100 µL) was transferred to an autosampler vial and 3.0 µL injected into a LC/MS system.

A calibration curve (0.05, 0.1, 0.25, 0.5 and 1.0 µg/mL) and quality control samples (0.08, 0.4 and 0.8 µg/mL) were spiked directly with diosmetin, without the deconjugation process. Therefore, these samples were not incubated with β-glucuronidase, but just diluted with 20 µL of water, 125 µL of acetate buffer (200 mM, pH = 4.6) and processed as above. Analysis was carried out on an UHPLC instrument (Thermo Scientific, UltiMate 3000, Thermo Fisher Scientific, San Jose, CA, USA) equipped with a binary pump, a degasser, an autosampler and a column compartment. Mass spectrometric analysis was carried out on an Orbitrap high-resolution mass spectrometer (Exactive TM; Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization (ESI) source and used for all high resolution mass spectrometry analysis.

The data acquisition was under the control of Xcalibur software version 2.1. The extracted ion chromatograms (EICs) were generated by extracting a small range (e.g. ± 5-10 ppm) centered on the exact *m/z* of each analyte. The separation of diosmetin and IS from endogenous compounds was achieved using a Gemini C18 column (50 x 2.1 I:D:; particle size 3.0 µm), Gemini C18 guard column (4 x 2.0 mm; Phenomenex, Torrance, CA, USA) and mobile phase consisting of water (A) and methanol (B) in a gradient mode: 0 – 1 min: 90% A; 1 – 4 min: 5% A; 4 – 8 min: 5% A; 8 – 10 min: 90% A; 10 – 15 min: 90% A. The flow rate of the mobile phase was 0.2 mL/min and column temperature 25°C. The retention times were approximately 6.7 min and 6.8 min for diosmetin and IS, respectively.

The operating source conditions for the MS scan in negative ion ESI mode were optimized as follows: sheath gas flow rate 35 au; aux gas flow rate 8 au; spray voltage 4.6 kV; capillary temperature 280°C; capillary voltage -52.5 V; tube lens voltage -65.0V; skimmer voltage -14.0 V and heater temperature 30°C. The theoretical monoisotopic *m/z* of each [M-H] was derived from the elemental composition. The full scan mode across *m/z* 294.1 – 304.1 (diosmetin) and 224 – 304 (IS) and EICs (*m/z* theoretical ± 5-10 ppm) was used. For quantification, EICs of [M-H] at *m/z* 299.05630 for diosmetin and *m/z* 229.01152 for IS with a 5ppm range centered on the exact *m/z* value were generated.

For diosmin concentration measurement, no enzymatic degradation procedure was used. Calibration curve (0.5, 1, 2.5, 5 and 10 µg/mL) and quality control samples (0.8, 4 and 8 µg/mL), were spiked directly with diosmin and processed as for diosmetin. The full scan mode across *m/z* 100 - 2000 was used. For quantification, EICs of [M-H] at *m/z* 607.16575 for diosmin with a 5 ppm range centered on the exact *m/z* value was generated.

Data evaluation: Non-compartmental analysis [10] was used to calculate the pharmacokinetic parameters using a MKModel by Nick Holford software Version 4 (Biosoft, Ferguson, MO, USA) or equivalent pharmacokinetic programs. The maximum (peak) plasma concentration (C_{max}) and the time to reach C_{max}(T_{max}) were the observed values obtained directly from the experimental data. The apparent elimination rate constant (k_{el}) was calculated as the negative slope of the log-linear terminal portion of the plasma concentration-time curve using linear regression. A minimum of 3 observations was used to calculate k_{el}. The half-life ($t_{½}$) was calculated as 0.693/k_{el}; the area under the plasma concentration-time curve (AUC_{last}) from time zero to the time of the last measured concentration (C_{last}) was calculated by the linear trapezoidal rule. The extent of relative oral bioavailability (F) was estimated by dividing AUCs obtained for the 2 different formulations. All data were expressed as mean ± standard deviation (SD) of 5 animals per group.

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