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Research Paper

Preclinical pharmacokinetics, dose proportionality, gender difference and protein binding study of 16-dehydropregnenolone, an antihyperlipidemic agent, in rats*

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Abstract

Objectives This manuscript addresses key pharmacokinetic issues in support of the development of a potent candidate lipid-lowering drug molecule, 16-dehydropregnenolone (DHP).

Methods Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) assay for simultaneous estimation of DHP and its metabolites, including 5-pregnene-3β-ol-16, 17-epoxi-20-one (M_1) was validated in male and female Sprague–Dawley rat plasma and applied to different studies. Pharmacokinetic studies of DHP after intravenous and oral administration were carried out to assess any gender effect. Dose-proportionality after oral administration was assessed at three dose levels. Protein binding was estimated using the modified charcoal adsorption method.

Key findings Rapid elimination of DHP from the systemic circulation resulted in a comparatively lesser systemic exposure in male compare to female rats. The area under the curve (AUC) after oral administration in males was significantly different tofemales. The large volume of distribution and low degree of protein binding suggest extensive distribution of DHP. An increase in the oral dose led to a disproportionate change in peak concentration (C_{max}) and AUC, indicating variable absorption. However, the dose-normalized AUC and C_{max} at two dose levels were not found to be statistically different.

Conclusions The extent of conversion of DHP to M_1 was higher after oral administration in male rats but was insignificant in female rats. DHP showed low systemic oral bioavailability and exhibited dose-independent pharmacokinetics and gender differences.

Keywords 16-dehydropregnenolone; dose proportionality; gender difference; pharmacokinetics; rats

Introduction

Clinical manifestations of atherosclerosis, such as myocardial infarction, stroke and peripheral vascular diseases, are associated with elevated low-density lipoprotein (LDL). It has been shown unequivocally that reducing plasma lipoprotein levels or, more accurately, decreasing the LDL high-density lipoprotein (HDL) ratio leads to beneficial effects.[1–3] In an attempt to produce a more potent and safer antihyperlipidemic agent, the Central Drug Research Institute of Lucknow, India (CDRI) developed 16-dehydropregnenolone (DHP, Figure 1) for the treatment of dyslipidaemia (Pratap *et al*., US patent, 1999, 09.280448; Nityanand *et al*., European patent, 1999, 99302556.8). Chronic toxicity studies have indicated that this drug is free from any untoward effects and possesses a good therapeutic window. DHP is a bile acid receptor (BAR) ligand and acts as a potent and efficacious BAR antagonist in cell-based assays.[4]

There is paucity of pharmacokinetic (PK) data on DHP, the exception being some preclinical pilot PK studies in rats and rabbits. The pilot PK data has been described as an application to the validation procedures.[5,6] The study of DHP in rats shows that levels of the compound are low after oral administration and also indicate 5-pregnene-3β-ol-16,17-epoxy-20-one (M_1, Figure 1) as being the major metabolite in plasma (unpublished data).

Preclinical pharmacokinetic studies in different animal species are an important step in the drug-development process. In the present study, pharmacokinetic experiments have been

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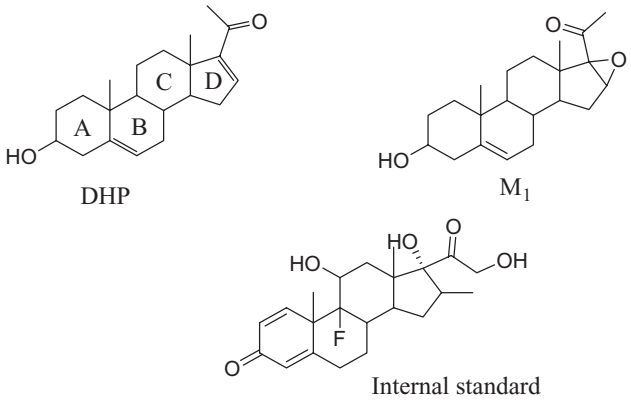

Figure 1 Chemical structure of DHP, M_1 and dexamethasone (internal standard).

carried out in rats, as they are physiologically most similar to humans. One of the prerequisites for successfully carrying out preclinical pharmacokinetic studies is the availability of a reliable, reproducible, accurate and precise bioanalytical method for monitoring the compounds under consideration. The liquid chromatography tandem mass spectrometry (LC-MS/MS) assay has been developed and validated in rabbit plasma for the simultaneous determination of DHP and its five putative metabolites.[6] In the present study, the assay was partially validated for estimation of DHP and M_1 in rat plasma (both male and female).

This paper addresses key pharmacokinetic issues, such as protein binding, bioavailability, dose proportionality and gender difference, in support of the development of DHP as a candidate drug.

Material and Methods

Chemicals and reagents

DHP (5,16-pregnadien-3β-ol-20-one) (purity > 99%) was synthesised at the Medicinal and Process Chemistry Division of CDRI, Lucknow, India. The reference standard of 5-pregnene-3β-ol-16,17-epoxi-20-one (M_1) (purity > 99%) was synthesised at the Pharmacokinetics and Metabolism Division, CDRI. Dexamethasone (internal standard) (purity > 99%) was purchased from HiMedia Laboratories Pvt. Ltd, Mumbai, India. High-performance liquid chromatography (HPLC) grade acetonitrile and isopropyl alcohol were procured from Thomas Bakers (Chemicals) Limited, Mumbai, India. n-Hexane (HPLC grade) was obtained from Spectrochem Pvt. Ltd, Mumbai, India. Ammonium acetate (AR grade) and glacial acetic acid (AR grade) were procured from E. Merck (India) Ltd, Mumbai, India. Heparin sodium injection I.P (1000 IU/ml) was obtained from Biologicals E. Limited, Hyderabad, India. Dextran-coated charcoal was procured from Sigma Chemicals, USA. Dulbecco's phosphate buffered saline (DPBS) (Ca^{2+}- and Mg^{2+}-free) was purchased from Hi Media Laboratories Pvt. Ltd, Mumbai. Ultra-pure water (18.2 MΩ^{-1} cm) was obtained from a Milli-Q Plus PF water purification system.

Prior approval (vide approval no. 58/08/PKM/IAEC/Renew 01 (61/09)) from the Institutional Animal Ethics Committee (IAEC) was sought for maintenance, experimental studies, euthanasia and disposal of carcasses of animals. Drug-free heparinised plasma was obtained from different young, healthy male/female Sprague–Dawley rats housed in the Laboratory Animal Services Division of the institute. Plasma samples were stored in glass tubes at −60°C until required.

LC-MS/MS analysis

An API-4000 (Applied Biosystems/MDS SCIEX, Toronto, Canada) mass spectrometer coupled with a Series 200 HPLC system (PerkinElmer, Norwalk, CT, USA) was used for quantitative analysis. Chromatographic separations were achieved on a Spheri-5 RP-18 column (5 μm, 100 × 4.6 mm i.d., Pierce Chemical Company, Rockford, USA) preceded by a guard column packed with the same material (30 × 4.6 mm i.d., 5 μm using isocratic mobile phase (acetonitrile:10 mM ammonium acetate buffer, 90 : 10 % v/v) at a flow rate of 0.65 ml/min. Analyst 1.4.2 software (Applied Biosystems/MDS SCIEX, Toronto, Canada) was used for control of the equipment, for data acquisition and for analysis. Zero air (atmospheric air purified to contain less than 0.1 ppm total hydrocarbons) was used as the nebuliser, while nitrogen was employed as the collision gas. The quantitation was performed in multiple reaction mode with an electrospray ionisation source. The parent–to-daughter ion transitions monitored were *m/z* 315.1 > 137.4, 348.4 > 271.5 and 393.2 > 171.1 for DHP, M_1 and internal standard, respectively. The plasma concentrations were calculated by reading the peak area ratios of the analytes and the internal standard with the calibration standard curve in plasma. Quality control (QC) samples (low, medium and high at 3.13, 50, 200 ng/ml) were incorporated after every six to eight unknown samples.

Sample cleanup

Sample preparation involved a simple two-step liquid–liquid extraction (2 × 2 ml) with distilled n-hexane : isopropyl alcohol (98 : 2 v/v). The extraction solution was added to 0.1 ml aliquots of blank/spiked plasma or the test samples and was vortex mixed (Type 37600 mixer, Thermolyne, Dubuque, Iowa, USA) for 60 s, then centrifuged (2000 rpm for 5 min) and the upper organic layer was transferred to another set of clean tubes by snap-freezing the lower aqueous layer with liquid nitrogen. The same process was repeated in the second step. The combined organic phase was evaporated under reduced pressure in a Savant Speed Vac (Farmingdale, NY, USA) at 40°C. The dry residue was reconstituted in 0.1 ml reconstituting solution (acetonitrile:10 mM ammonium acetate buffer, 90 : 10 v/v). The samples (20 μl) were injected onto the LC-MS/MS system.

Validation parameter

The LC-MS/MS method was partially validated for 3 days with five replicates of each QC sample at three levels (low, medium and high at 3.13, 50 and 200 ng/ml, respectively) to determine linearity, selectivity, sensitivity, recovery, accuracy and precision.[7,8]

Plasma protein binding

Protein binding was estimated using the modified charcoal adsorption method.[9]

Preparation of dextran-coated charcoal suspension

The dextran-coated charcoal (0.66 g) was transferred to a 250 ml reagent bottle containing 100 ml of DPBS and the mixture was stirred with a magnetic stirrer at room temperature until the charcoal was suspended. This suspension was prepared at least 18 h before use and stored at 5–10°C for not longer than 30 days. The stored charcoal mixture was resuspended before use.

Charcoal adsorption assay

Male and female rat plasma (6.0 ml) was spiked with 60 μl of DHP to obtain a concentration of 5 μg/ml. The spiked plasma was allowed to equilibrate for 10 min before the start of the study. The charcoal suspension (6.0 ml) was transferred into a 30-ml glass tube, centrifuged at 3000 rpm for 15 min at 25°C, and the supernatant DPBS was carefully decanted off. Spiked plasma (6.0 ml) was then added onto the charcoal pellet under continuous stirring at 37 ± 2°C, with the temperature maintained using an oil bath. Serial samples (200 μl) were withdrawn at 0 and 5 min, and then at 0.25, 0.5, 0.75, 1.0, 1.5, 2, 4, 6 and 8 h in a 0.6 ml eppendorff tube and centrifuged immediately at 11 000 rpm and 37°C. The supernatant was separated and was immediately transferred into a 5 ml glass tube and stored at −60°C until analysed.

Pharmacokinetic study following intravenous and oral administration

Animals

Young, adult male/female Sprague–Dawley (SD) rats, weighing 225–250 g, were procured from the National Laboratory Animal Center, CDRI (Lucknow, India). Rats were housed at the Pharmacokinetics & Metabolism Division, CDRI, in well-ventilated cages at room temperature (24 ± 2°C) and 40–60% relative humidity, while on a regular 12 h light–dark cycle. The animals were acclimatised for a minimum period of 3 days prior to the experiment. Standard pellet rodent chow (Goldmohar Laboratory Animal Feed, Chandigarh, India) and water were freely available. Animals in the oral dose group were fasted for 8–12 h before dosing, but allowed free access to water. Approval from the Local Animal Ethics Committee was sought and the study protocols were approved before the commencement of the studies.

Drug formulation and administration

The intravenous formulation was a clear solution of DHP in dimethylacetamide, propylene glycol and normal saline at a ratio of 10 : 50 : 40 (v/v/v). The intravenous formulation was sterilised by filtration before use. For intravenous treatment, a single bolus dose was injected into the tail vein of the rats. The volume administered was less than 1 ml/kg (0.8 ml/kg, i.e. a 250 g rat received 0.2 ml). The dose proportionality study was carried out at an equivalent therapeutic dose of DHP in male rats, i.e. 72 mg/kg and ± $^1/_2$D (~36 and 108 mg/kg). The upper and lower dose was determined based on the poor solubility profile of DHP and possible dose adjustment. To study the gender-specific pharmacokinetics of DHP, an intravenous dose (1 mg/kg) and oral dose (72 mg/kg) was administered in female rats. Aqueous suspension of DHP using carboxy methyl cellulose (0.05% w/v) as suspending agent was developed as the oral formulation in all cases. The volume factor for oral administration was 2 ml/kg at all the dose levels. The suspensions were well stirred to ensure content uniformity for the successively drawn doses and administered using a 20 G gavage needle.

Sampling schedule

The pharmacokinetic studies were carried out using the sparse sampling approach, wherein each time point is based on samples from three different rats ($n = 3$) and each rat contributes two time points. The first blood sample was collected by cardiac puncture (~0.25 ml) followed by terminal sampling from the inferior vena cava.[10] The total volume of blood withdrawn within 24 h through cardiac puncture was less than 5% of the total blood volume. Blood samples were transferred to heparinised tubes (22 IU heparin per millilitre of blood) at 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 18, 24 and 48 h post dose in the case of oral administration. Following intravenous administration, three early time points, at 0.083, 0.17 and 0.33 h, were also included in the sample collection schedule. Plasma was separated by centrifugation at 2000 rpm for 10 min and stored at −60°C prior to analysis.

Data analysis

For the protein binding estimation, the data for the percentage of the drug remaining in the supernatant plasma versus time was fitted to a two-compartment model against intravenous bolus input, which mimics the events pertaining to the charcoal adsorption assay.[9] The model is described by following biexponential equation:

$$B_t = A_1 e^{-\alpha t} + A_1 e^{-\beta t}$$

where B(t) is the percentage bound at time t, A_1 and A_2 are the y intercepts and α and β are the distribution and disposition rate constants for the two phases, respectively. The extent of protein binding is given by comparing the C_0 value extrapolated from the model with the observed initial concentration.

The plasma concentration–time data after oral and intravenous administration were subjected to non-compartmental analysis using statistical moment theory.[11] The highest observable concentration and the associated time point were defined as the peak concentration (C_{max}) and time to peak concentration (t_{max}). Semilogarithmic plots of the concentration–time data were examined to determine the appropriate data point for estimating the terminal elimination rate constant (λ_Z). The area under the plasma concentration–time curve from time zero to the last quantifiable concentration (AUC_t) was calculated using the linear trapezoidal rule. $AUC_{0-\infty}$ was calculated as the sum of AUC_t and C_t/λ_Z, where C_t represents the last quantifiable concentration. Clearance (CL) was calculated by dividing the dose by $AUC_{0-\infty}$ for intravenous administration. Mean residence time (MRT) was determined by dividing the area under the first moment curve

($AUMC_{0-\infty}$) by $AUC_{0-\infty}$. The apparent volume of distribution (V_d) was given by the quotient between CL and the elimination rate constant λ_Z. The volume of distribution at steady state (V_{ss}) was calculated as the product of the CL and MRT_{iv}. The mean absorption time (MAT) was calculated by subtracting the MRT_{iv} from MRT_{oral}. The percentage conversion of DHP to its major metabolite M_1 was calculated using following equation:

$$\%\text{conversion} = \frac{AUC_{\text{metabolite}}}{AUC_{DHP}} \times 100$$

The absolute bioavailability ($\%F_{oral}$) was determined using following equation:

$$\%F_{\text{absolute}} = \frac{AUC_{\text{oral}} \times \text{dose}_{\text{i.v.}}}{AUC_{\text{i.v.}} \times \text{dose}_{\text{oral}}} \times 100$$

Statistical analysis of data

The mean ± SEM values for the dose-normalised AUC and C_{max} of DHP at two dose levels (72 and 108 mg/kg) in male rats were compared using the unpaired Student's *t*-test to check the dose proportionality. The mean ± SEM values for AUC of DHP in male and female rats after a 72 mg/kg dose were compared using the unpaired Student's *t*-test to measure significant differences in DHP pharmacokinetics.

Results

Method validation

Linearity, selectivity, sensitivity, recovery, accuracy and precision were measured and used as the parameters for assessment of the assay performance. The peak area ratios of the analytes to the internal standard in rat plasma were linear over the concentration range 1.56–400 ng/ml for both analytes. The calibration model was selected based on the analysis of the data by linear regression with and without intercepts ($y = mx + c$ and $y = mx$) and weighting factors (1/x, $1/x^2$ and 1/log x). The best fit for the calibration curve was achieved by a linear equation of $y = mx + c$ and $1/x^2$ weighting factor for both analytes. The correlation coefficient for both analytes was above 0.996 over the concentration range used.

LC-MS/MS analysis of the blank plasma samples showed no interference with the quantification of both analyte and internal standard (Figure 2). The specificity of the method was established with pooled and individual plasma samples from eight different sources. The retention times of all the analytes and the internal standard showed little variability with a relative standard deviation (RSD) well within the acceptable limit of 5%.

The limit of detection for both analytes was 0.78 ng/ml, with a signal-to-noise ratio ≥ 3. The lowest limit of quantitation (LLOQ) for DHP and M_1, from normal rat plasma and with acceptable accuracy and precision, was established as 1.56 ng/ml. The LLOQ was established with five samples independent of the standard curve.

Recovery was calculated from the average peak area of the processed QC samples read against those of analytical standards fortified with blank extracted. Overall recovery corresponds to the net response after subtraction of the ion suppression and the signal loss due to the extraction. These experiments were performed at three concentration levels (low, medium and high) in triplicate. The average recoveries for DHP and M_1 were 87.48 ± 4.05 and 85.26 ± 5.16%, respectively.

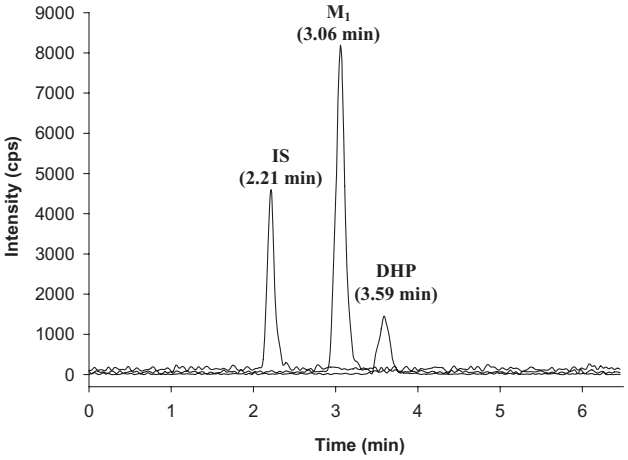

Figure 2 TIC chromatogram of DHP, M_1 and internal standard (IS) in fortified blank rat plasma overlaid with extracted blank male or female rat plasma.

The accuracy and precision (intra- and inter-day) were calculated with three QC samples in triplicate for three different days and are presented in Table 1. The precision was determined by one-way ANOVA as intra- and inter-day percentage RSD. Accuracy was expressed as percentage bias. The results show that the method is accurate since the bias is within the acceptable limits of ± 20% of the theoretical value at LLOQ and ± 15% at all other concentration levels.[7,8] The precision around the mean value never exceeds 15% at any of the concentrations studied.

Plasma protein binding assay

The percentage binding is then estimated from the decline of percentage of the drug remaining in the supernatant after the addition of charcoal. Figure 3 depicts the percentage of DHP remaining (mean ± SEM, $n = 3$) versus time after the addition of male and female rat plasma containing 5 μg/ml of DHP onto the charcoal pellet. The percentage protein binding in male and female rats was found to be 16.26 ± 0.5% and 14.35 ± 0.35%, respectively.

Intravenous and oral dose pharmacokinetics in male Sprague–Dawley rats

The plasma concentration–time profile (mean ± SEM, $n = 3$) and PK parameters of DHP following intravenous administration in male rats are shown in Figure 4 and Table 2, respectively. The levels of DHP could be determined up to 5 h. The elimination half-life, $t_{1/2}$, and $MRT_{i.v.}$ were found to be 2.13 and 2.45 h, respectively. The $AUC_{0-\infty}$, plasma clearance and volume of distribution at steady state (V_{ss}) obtained with intravenous administration were 79.44 ng.h/ml, 12.59 l/h/kg and 38.75 l/kg, respectively.

Table 1 Accuracy and precision of assay of DHP and its metabolite M_1 in male and female rat plasma

Matrix	Analytes	Concentration (ng/ml)	Accuracy (%bias)[a]		Precision (%RSD)[a]	
			Inter-day	Intra-day	Inter-day	Intra-day
Male rat plasma	DHP	3.13	7.79	3.59	10.1	3.2
		50	4.31	–4.61	5.0	7.2
		200	–1.50	0.79	7.4	5.8
	M_1	3.13	3.3	4.4	14.0	7.4
		50	–4.9	3.0	9.2	8.4
		200	2.3	0.30	8.7	8.1
Female rat plasma	DHP	3.13	4.84	2.41	6.2	6.4
		50	1.74	1.75	6.1	10.0
		200	5.06	4.48	3.7	3.3
	M_1	3.13	–2.21	–1.99	8.5	7.0
		50	6.20	2.84	5.2	2.5
		200	2.19	2.18	5.2	5.6

[a]Accuracy and precision were calculated on three different days. RSD, relative standard deviation.

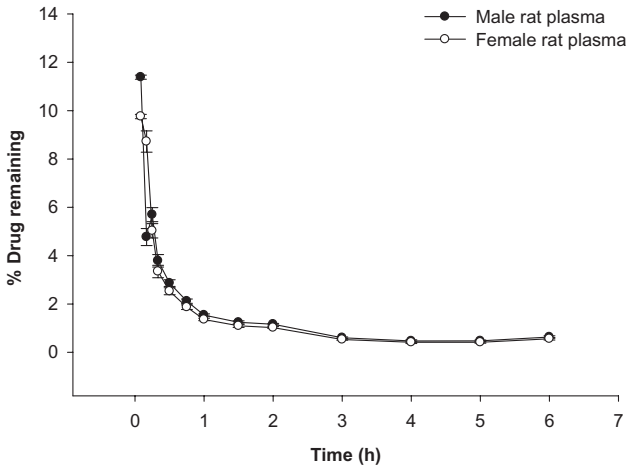

Figure 3 Decline of DHP in male rat plasma and female rat plasma at 5 μg/ml spiked concentration using charcoal adsorption method. Figures shown as mean ± SEM, $n = 3$.

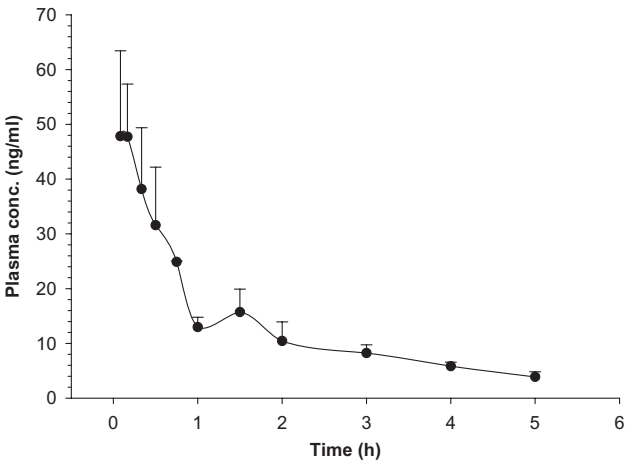

Figure 4 Plasma concentration–time profile of DHP following intravenous dose administration. Dosing at 1 mg/kg in male Sprague–Dawley rats. Figures shown as mean ± SEM, $n = 3$.

The oral pharmacokinetic study was carried out at three doses: 36, 72 and 108 mg/kg. At 36 mg/kg, the systemic levels of the parent compound and its metabolites were erratic and below the lowest limit of quantitation for most of the time points, therefore the concentration–time profile could not be generated at this dose. The time courses of plasma concentration (mean ± SEM) of DHP obtained after oral administration of 72 and 108 mg/kg doses to male rats are presented in Figures 5 and 6 and detailed pharmacokinetic parameters are given in Table 2. After 72 and 108 mg/kg oral doses, visual examination of the data indicated the presence of multiple peaks at t_0, 0.5, 1.5 and 4 h in the plasma concentration–time profile of DHP. The elimination $t_{1/2}$ and absolute bioavailability (%F) of DHP were 2.74 h and 2.93% at 72 mg/kg and 4.78 h and 2.31% at 108 mg/kg, respectively.

Oral and intravenous dose pharmacokinetics in female Sprague–Dawley rats

Figure 7 depicts the mean ± SEM plasma concentration–time profiles of DHP following intravenous and oral administration. The pharmacokinetic parameters are summarised in Table 2. After intravenous administration, the plasma concentration of DHP declined bi-exponentially and levels could be detected up to 18 h. $AUC_{0-\infty}$, CL and elimination $t_{1/2}$ were found to be 153.52 ng.h/ml, 6.46 l/h/kg and 4.46 h, respectively. Irrespective of gender, large V_d (41.93 l/kg) suggests DHP's extensive distribution in the body.

After oral administration, DHP was rapidly absorbed to the systemic circulation as evident from early peak plasma concentrations, with t_{max} reached within 0.5 h. The secondary C_{max} values were not significant in female rats. After oral dosing V_d (57.73 l/kg) and elimination $t_{1/2}$ (6.19 h) were higher than with intravenous dosing. The absolute oral bioavailability of DHP in female rats was significantly low at 0.59%, compared to male rats.

Pharmacokinetics of metabolites

In male rats after oral administration, conspicuous plasma concentrations of M_1 were present. However these concentrations were below the limit of quantitation after intravenous

Table 2 Pharmacokinetic parameters for DHP and its major metabolite M_1 in rats after a single-dose administration

Pharmacokinetic parameter (units)		Male rat					Female rat[a]	
		Intravenous (1 mg/kg)	Per-oral[b]				Intravenous (1 mg/kg)	Per-oral (72 mg/kg)
			72 mg/kg		108 mg/kg			
			DHP	M_1	DHP	M_1		
$AUC_{0-\infty}$ (ng h/ml)		79.44	167.92	203.95	197.97	35.16	153.52	65.70
AUC (ng h/ml)[c]		67.53 ± 10.5	157.04 ± 25.17*	198.45 ± 79.65	189.1 ± 37	31.04 ± 4.16	144.57 ± 40.13	46.79 ± 5.03*
C_{max} (ng/ml)[d]	1	–	14.75 ± 4.18	–	8.86 ± 0.1	3.78 ± 0.8	–	14.49 ± 8.04
	2	–	21.95 ± 7.72	40.18 ± 30.5	11.45 ± 1.2	2.57 ± 0.6	–	–
	3	–	27.24 ± 10.16	46.37 ± 33.8	47.5 ± 14.5	8.51 ± 2.14	–	–
T_{max} (h)[d]	1	–	0.5	–	0.5	0.5	–	0.5
	2	–	1.5	1.5	1.5	1.5	–	–
	3	–	4	4	4	4	–	–
$t_{1/2}$ (h)		2.13	2.74	2.02	4.78	2.18	4.46	6.19
AUMC (ng.h^2/ml)		195.03	960.94	926.40	1549.15	194.10	778.32	527.62
CL (l/h/kg)		12.59	12.56	–	12.61	–	6.51	6.46
V_d (l/kg)		38.75	49.72	–	86.95	–	41.93	57.73
MRT (h)		2.45	5.72	4.54	7.83	5.52	5.06	8.03
%F		–	2.93	–	2.31	–	–	0.59

–, Not applicable; AUMC, area under moment curve; CL, clearance; MRT, mean residence time; %F, bioavailability. [a]Only DHP PK parameters are presented. Metabolite levels were not detectable in female rats. [b]PK parameter at 36 mg/kg could not be reported due to erratic and systemic levels of DHP below LLOQ. [c]Area under the curve (AUC) from time 0 to last observed concentration. [d]$C_{max(1,2,3)}$ and $T_{max(1,2,3)}$ are presented as mean ± SEM ($n = 3$), where number 1, 2, 3 corresponds to the three different peaks. *Statistically significant, $P < 0.05$ (Student's *t*-test). AUMC, ;CL, ; V_d, ; MRT, ; %F, .

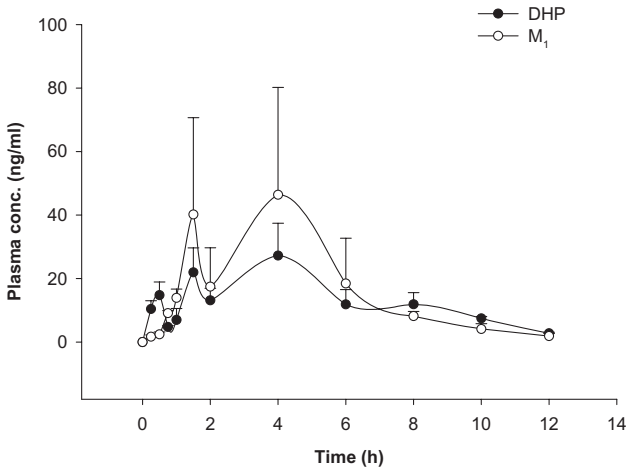

Figure 5 Plasma concentration–time profile of DHP and its metabolite M_1 following oral administration at 72 mg/kg. Male Sprague–Dawley rats. Figures shown as mean ± SEM, $n = 3$.

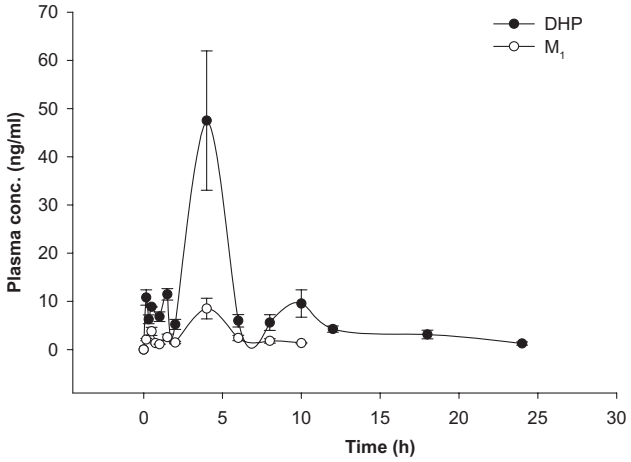

Figure 6 Plasma concentration–time profile of DHP and its metabolite M_1 following oral administration at 108 mg/kg. Male Sprague–Dawley rats. Figures shown as mean ± SEM, $n = 3$.

dosing. The plasma concentrations–time profiles of M_1 after oral administration are shown in Figures 5 and 6. Peak plasma concentrations of 46.37 and 8.51 ng/ml were reached 4 h after oral administration of 72 and 108 mg/kg of DHP, respectively. The extent of conversion of DHP to M_1 was higher with the 72 mg/kg dose. In female rats, the plasma levels of metabolite M_1 after oral and intravenous administration were found to be below the LLOQ.

Discussion

The traditional techniques for the determination of protein binding cannot be used for DHP because of its extensive non-specific adsorption to the dialysis cell and various parts of ultrafiltration devices. A charcoal adsorption assay specifically designed for these types of compounds was therefore used in the present study.

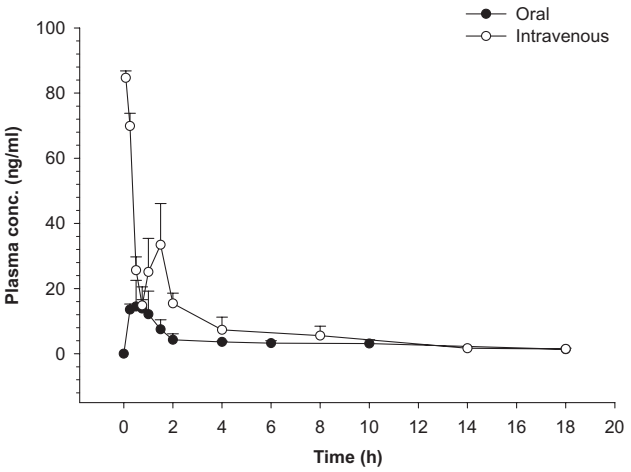

Figure 7 Plasma concentration-time profiles of DHP following oral and intravenous dosing in female rats. Oral dosing at 72 mg/kg and intravenous dosing at 1 mg/kg in female Sprague–Dawley rats. Figures shown as mean ± SEM, $n = 3$.

Due to solubility constraints a single low dose level (1 mg/kg) was selected to generate intravenous pharmacokinetic data. Following intravenous administration, the elimination of DHP from the systemic circulation was rapid, as evidenced by its high systemic clearance and relatively short elimination half-life. The large V_{ss} suggests that DHP undergoes extensive distribution. The low degree of protein binding (~16%) in blood also underlines this observation.[12] The shorter elimination half-life of metabolite M_1 (~2.0 h) suggests that DHP is likely to be cleared by the metabolism by forming metabolic products that are more hydrophilic and can therefore be eliminated more readily.

After oral administration, the presence of multiple plasma peaks at 0.5, 1.5 and 4 h might be suggestive of absorption of DHP from distinct regions of the alimentary tract, i.e. the small intestine and ileocolonic region, an idea that matches with the transit time of fluid from the duodenum to the colon, i.e. 3 to 4 h.[13] Generally, the mechanisms proposed to explain multiple peak phenomenon include biphasic dissolution, site-specific absorption and enterohepatic recycling,, as well as other physiological phenomena.[14–16] In the present study, low solubility (<1 μg/ml in simulated gastric fluid) of DHP may also be contributing to the appearance of multiple peaks.[17] The V_d of the compound was larger with the oral than the intravenous route and CL values exceeding the hepatic blood flow suggest that DHP might be poorly absorbed or undergoes extensive first-pass or presystemic elimination.[18,19] An increase in the oral dose led to disproportionate changes in peak concentration (C_{max}) and area under the curve (AUC): at the higher dose level (108 mg/kg), dose-normalised AUC (~1.83) decreased by 22% compared to the 72 mg/kg dose (~2.33), indicating variable absorption. However, dose-normalised AUC and C_{max} at two dose levels (72 and 108 mg/kg) were not found to be statistically different ($P < 0.05$). Dose-dependent pharmacokinetics are commonly reflected in greater than or less than proportional increases in AUC with an increase in dose.[20] In the present study design ($n = 3$), the

absence of such trends indicates dose-independent pharmacokinetics for DHP in rats at the dose levels studied.

After intravenous administration in female rats, DHP cleared rapidly from the systemic circulation but slower than in males (6.46 vs 12.59 l/h/kg), resulting in greater systemic exposure (153.52 vs 79.44 ng.h/ml). After oral dosing DHP absorbed rapidly, but plasma levels were low enough at distribution phase might causes the absence of secondary C_{max} in female rats. The dose-normalised AUC after oral administration of DHP in females decreased by 39% (significantly different, with $P > 0.05$) compared to male rats.

Conclusions

In conclusion, an accurate and precise LC-MS/MS assay was successfully applied to generate pharmacokinetic data in male and female Sprague–Dawley rats. DHP distributed extensively and was rapidly cleared from the systemic circulation. It showed low systemic oral bioavailability in male and female rats. DHP exhibits dose-independent pharmacokinetics in rats at the studied dose levels. Furthermore, significant differences were observed in the data for male and female rats, reflecting the presence of gender differences in the pharmacokinetics of DHP.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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