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Comparison of Pharmacokinetics of Dapsone in Male Sprague Dawley Rats Following Retro Orbital, Jugular Vein and Saphenous Vein Blood Sampling

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Abstract

Pharmacokinetic (PK) studies play an important role in identifying lead compounds for further development. Typically rats are used for PK screening of New Chemical Entities (NCEs) as the compound requirements are minimal (<10 mg), multiple blood sampling (up to 10 samples) can be performed from the same animal and in small volumes (5-25µL) for sample analysis. Blood sampling site is critical in obtaining multiple blood samples of good quality and in small volumes with minimal stress to animals. However, it is not known whether PK parameters can be influenced by sampling site. Thus, in this study, we evaluated the effect of different blood sampling sites like retro-orbital plexus, jugular vein and saphenous vein on PK parameters of Dapsone. Dapsone was administered both orally and intravenously at a dose of 12 mg/kg to a group of 4 male Sprague Dawley rats and blood samples were collected up to 24 h. Samples were analyzed by LC/MS/MS and PK parameters were calculated. With all the sampling techniques, PK parameters like clearance, volume of distribution, half-life and bioavailability were similar. Due to the control on the blood volume withdrawn at each time point, quick sampling with minimal hemolysis and minimal animal handling stress during sampling, Jugular Vein (JV) or Saphenous Vein (SV) sampled rats can be used for PK studies. Further for saphenous sampling no pre-study preparation like cannulation is required before dosing the animals therefore sampling of rats through saphenous vein is recommended for pharmacokinetic and toxicokinetic studies. To summarize, SV sampling reduce the number of animals in different Pharmacokinetic (PK) (mouse) and Toxicokinetic (TK) (mouse and rat) studies by using serial draws, offers reduction and refinement over the othersampling techniques with minimal preparation upfront and with a potential to replace them.

Keywords: Pharmacokinetics; LC-MS/MS; Retro-orbital sampling; Jugular vein cannulation; Saphenous vein sampling

Abbreviations

AUC: Area Under the Curve; C_{max}: Concentration maximum; NCEs: New Chemical Entities; IV: Intravenous; JVC: Jugular Vein Cannulation; PO: Per Oral; PK: Pharmacokinetics; SV: Saphenous Vein; LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry

Introduction

Pharmacokinetic (PK) studies play a vital role in selecting a lead compound for further development. Rats are the preferred rodent species for assessing PK behavior of new chemical entities (NCEs).Typically during drug discovery phase limited compound availability can be a hurdle to conduct detailed and robust pharmacokinetic studies with large number of animals. In addition, using large number of animals is of ethical concern and therefore complying with 3Rs (reduce, refine and replace) is of major challenge. The recent advances in micro sampling techniques, highly sensitive LC-MS methods made it possible to conduct robust and detailed PK studies during drug discovery, with limited number of animals.

In vivo PK studies in rodents like hamster, mouse and rat remain a preferred screening strategy for selecting lead molecules for development. Typically, in PK studies, following administration of the compound, serial blood samples (usually 9-10 time points) are preferred, as they give a more robust concentration versus time profile within the same animal. These samples are analyzed for the parent or metabolite using a suitable analytical technique. The objective is to determine the rate of appearance and/or disappearance of the compound in the blood/plasma. Based on the PK and other druggable properties, the NCEs are rank ordered and decisions are made for further development of the NCE.

There are various factors which can affect the quality of PK data such as stress during animal handling [1], blood loss with serial sampling [2], feed [3,4], age and gender [5]. Another important factor to consider is blood sampling site like retro-orbital puncture [6], tail vein [6], Saphenous vein [6,7], jugular vein [8-10], sublingual [11], and tail snip [12], all of which have their own inherent advantages and disadvantages. According to published literature, in a day (24 h), no more than 10% of total

circulating blood volume should be withdrawn from each animal [13,14]. For example, from a rat weighing 250 g containing ~16mL blood, a maximum of 1.6 mL of blood can be withdrawn in 7 day period. Thus, only 160 µL of blood can be withdrawn for each time point for a 10 time point serial blood collections and only 70-80 µL of plasma can be harvested from it. The recent advances in analytical methods permit the use of plasma sample as low as 5 µL for quantitation of the analyte [15].

Of the different sampling techniques, retro-orbital puncture is the most preferred method of blood sampling but controlling blood flow to below 160 µL blood withdrawn per time point is a challenge. In addition, it is recommended that blood sampling via this route is conducted under general anesthesia. However, some anesthetic agents undergo biotransformation by cytochrome P450 isozymes and inactivate CYP enzymes, thus leading to flawed PK estimations. After using anesthesia, artificial tears are recommended to offset the dryness of eyes. In addition, retro-orbital puncture may also be subjected to potential ocular complications including hematoma, corneal ulceration, keratitis, pannus formation, rupture of the eye globe, damage of the optic nerve and other intra-orbital structures and necrotic dacryoadenitis [16], of the harderian gland [17-20]. Due to these reasons, Netherlands banned retro-orbital blood sampling.

Lateral tail vein bleeding is also used very often for pharmacokinetic studies. Repeated blood sampling via tail vein, does not require anesthesia, and low sample volumes (100-150 µL/time point) can be obtained. Repeated punctures of tail vein at the same site may result in bruising, blood clots and necrosis. Since tails of the animals are used to lift the animals any damage to it induces further stress in animals. Thus, it is recommended to use different sites of the vein on the tail starting from tip of the tail to the tail base.Another frequent problem is warming of tail vein to improve blood flow and visibility of the vein. This can lead to dehydration and increasing the metabolic rate. During pharmacokinetic sampling of earlier time points, it can create problems in accurate timing of obtaining blood samples. In addition, animals also require a restrainer. It is also reported that the blood obtained from tail vein may be of poor quality and often hemolyzed with increase in stress levels as indicated by increase in blood glucose levels [21,22].Tail vein bleeding may also be conducted by amputation of tail tip. However, serial amputation resulting in a significant shortening of the tail, (i.e. > 5mm) are not acceptable. In addition, this technique is not suitable for older animals.

Another popular and often preferred method of PK blood sampling is by cathetered jugular vein. This technique requires prior surgical manipulation for implanting the catheter. Animals require general anesthesia for this procedure and conducted aseptically. After surgery, animals require 48 h of recovery period. Analgesics like buprenorphine with short half-life are usually given to animalsto reduce the pain from surgery [23]. However, these analgesics are extensively metabolized in liver by CYP3A4 and CYP2D6 and care should be exercised to prevent potential flawed PK results due to drug-drug interaction potential. It is essential that animals do not contract infection during the recovery period and that the cannula is firmly implanted without any blood clots.The advantage of using these animals is that fluid can be replenished so that blood samples can be taken without hypovolemic shock. During the course of the experiment, there is no need to handle the animal at any time point and sampling is done through a catheter.

Another technique for repeat blood sampling in rats and mice is via saphenous vein. This technique does not require anesthesia but requires animal holding. The saphenous vein is on the lateral side of the tarsal joint and easier to see when the fur is shaved and wiped with alcohol. The vein is raised by gentle pressure above the joint and the vessel is punctured using the smallest gauge needle (e.g. 25-27 g) that enables sufficiently rapid blood withdrawal without hemolysis. For small volumes (~10-15 µL), a simple stab leads to a drop of blood forming immediately at the puncture site and a hematocrit tube can be used to collect a standard volume. At the site of collection silicon greaseis applied to ensure that the blood sample does not spread [7]. Removal of the clot scab enables serial sampling. Thus, serial sampling with controlled blood flow is an advantage with this technique. After blood has been collected, pressure over the site is sufficient to stop further bleeding.

In this study, using dapsone as the model drug [24-27], we compared its pharmacokinetic behavior following oral and intravenous administration and blood sampling via retro-orbital plexus, jugular vein and Saphenous vein [25]. The results are discussed with regard to the sampling site and pharmacokinetic behavior of dapsone.

Experimental

Chemicals and reagents

Dapsone (catalogue # 46158, purity 100%), midazolam (catalogue # M2419, purity: 99%), ammonium formate (catalogue # 17843, purity > 99%) and formic acid (catalogue # V800192, purity > 98%), were procured from Sigma-Aldrich (Bengaluru, India). HPLC grade acetonitrile and methanol were procured from (Merck, Mumbai, India). All other reagents used in the study were of analytical reagent (AR) grade.Polyethylene (PE)-10, PE-30, PE-50 and tygon tubing were purchased from Instech, Ahmedabad, India. Sterile water for preparation of formulations was procured from medical store.

Animal husbandry

All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) of Advinus Therapeutics Limited (an AAALAC accredited facility), Bengaluru and were in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Male Sprague Dawley rats (8-12 weeks) weighing between 250-300 g were procured from in-house animal facility. Rats were housed individually in polypropylene cages. Temperature and humidity were recorded daily and were maintained between 22±3°C and 40-70%, respectively, with 12 h light and dark cycle. All the animals were acclimatized to the experimental conditions for 5 days before dosing. Animals catheterized with jugular vein

were given a recovery period of 48 h before dosing. Ssniff® rodent pellet diet (SsniffSpezialdiäten GmbH, Germany) and water (UV treated and purified through water filter) were provided *ad libitum*. Animals in the oral group were fasted overnight and food was provided 4 h post dose.

Cannula construction for jugular vein catheterization

Hard cannula (PE-50, 13 cm) and a microbore tygon tube of 2 mm were linked with a bio-adhesive and allowed to dry for 15 min. Polyurethane soft cannula of approximately 1.5 cm was cut and allowed to dilate by dipping in xylene, then inserted into PE-50 cannula (approx. 4 to 5 mm) and ensured both are tightly bound. The cannula was washed with water and sterilized it by placing in 70% ethanol overnight. The cannula was cut to have 3 cm length from the insertion end to bead of the cannula.

Jugular vein cannulation

Rats were anesthetized by using ketamine and xylazine mixture (90:10, IP, Dose volume: 2 mL/kg). A 2 cm ventral cervical skin incision was made right of the midline. Underlying salivary and lymphatic tissues were separated by means of blunt dissection to visualize the right common jugular vein. Jugular vein was then isolated from surrounding tissues and pair of thread was passed below the blood vessel. Tunnel was made with help of trochar to exteriorize the cannula towards neck. The jugular vein catheter consisted of polyethylene (PE-30) as catheter tippet and polyurethane (3 Fr) cannulas catheter body. The exteriorized part was made secure in place with the 3-0 life line sterile thread. Skin incision was closed and the exteriorized cannula was filled with lock solution (100 IU/mL of heparinized saline). Animals were then kept on a thermo pad maintained at 37°C to recover. With this procedure, we achieved patency of 100% and were maintained for at least 72 h without any blockages.

Study design

Animals in each group were dosed as per the study design presented in table 1. All the animals were weighed before dose administration and were used for calculation of volume required for each animal. Dose formulations were prepared on the day of dosing. Formulation recipe consisted of 5% v/v ethanol and 25% PEG 300and sterile waterfor injection q.s., for both intravenous and oral dose administration.Before dosing, an aliquot (100 µL) of formulations were collected in 100 µL of acetonitrile in triplicate. Formulations for each group were analyzed by LC/MS/MS and were found to be within ±15% of nominal concentration. Intravenous dosing for all the group animals was performed through tail vein and oral dosing was performed using gavage needle.

Sampling

Blood samples were collected at pre-dose, 0.083 (only IV), 0.25, 0.5, 1, 2, 4, 8 and 24 h in microfuge tubes containing K_2EDTA (20 µL/mL of blood, 200 mM) as anticoagulant. For retro-orbital sampling, animals were anesthetized with isoflurane. In jugular vein catheterized rats, after each sampling, equal volume of heparinized saline (10 IU/mL) was injected. For collection of blood in saphenous vein animals, the sampling area was shaved and applied with silicon grease. Plasma was harvested from blood by centrifugation of samples at 2500 g for 10 min at 4°C and stored below -60°C until bioanalysis.

Bioanalysis

All samples were processed using protein precipitation method and analyzed using LC-MS/MS (LC System, Shimadzu; API-4000, AB SCIEX) method employing positive ionization ESI mode. The chromatographic separation was carried out on a C-8 reverse phase column (Kromasil, 50 x 4.6 mm, 5 micron; Phenomenex, Hyderabad, India). The mobile phase consisted of acetonitrile:ammonium formate (60:40) containing 0.05% formic acid at a flow rate of 0.5 mL/min. The column oven was maintained at 30°C. The analyte (dapsone) and midazolam (internal standard) samples were monitored using multiple reaction monitoring (MRM) transitions of 249.20/156.10 m/z and 326.10/291.10 m/z, respectively. The optimized LC-MS/MS parameters had declustering potential of 80 V, entrance potential of 10 V, collision energy for MS/MS was 20 eV, collision gas was 10 Psi, curtain gas was 30 Psi, ion gas 1 was 30 Psi, ion gas 2 was 60 Psi, ion spray voltage was 3000 V and temperature was 500°C. All samples collected from the jugular vein, retro-orbital and saphenous vein were diluted 5-10 fold. An aliquot of plasma (50 µL) was processed by addition of 200 µL of internal standard containing midazolam (25 ng/mL). All the samples were vortex mixed for 5 min and centrifuged at 2500 g for 5 min. The supernatant was transferred to the LC-MS/MS vials and analyzed. The method employed a calibration curve range of 0.48 to 5970 ng/mL for dapsone with at least 8 non-zero calibration standards with acceptance criteria of ±15% at all concentrations, except ±20% at LLOQinterspersed with quality control samples consisting of LQC, M1QC, MQC and HQC. Dilution quality control samples were also assessed to ensure the dilution integrity.

Pharmacokinetic analysis

Pharmacokinetic parameters were calculated using non-compartmental analysis tool of validated Phoenix WinNonlin® software (Version 6.3). The area under the concentration time curve (AUC_{last} and AUC_{inf}) was calculated by linear trapezoidal rule. Peak plasma concentration (C_{max}) and time for the peak plasma concentration (T_{max}) were observed values. The C_0 was estimated following intravenous bolus dose administration by back-extrapolating the first two concentration values. The clearance (CL) and volume of distribution at steady state (V_{ss}) following intravenous administration were predicted values. The elimination rate constant value (k) was calculated by linear regression of the log-linear terminal phase of the concentration-time profile using at least 3 declining concentrations in terminal phase with a correlation coefficient of > 0.8. The terminal half-life value ($T_{1/2}$) was calculated using the equation 0.693/k. The absolute bioavailability was calculated using dose normalized AUC_{inf} of oral to that of intravenous.

Statistical analysis

Exposure (C_{max} and AUC_{inf}) between different groups for

IV and PO dose administration were compared for statistical significance using a one-way Analysis of Variance (ANOVA) with Tukey's multiple comparisons set. A P value of less than 0.05 was considered significant. The statistical analysis was performed using GraphPad Prism software (5.2).

Results

A summary of different sampling techniques used for pharmacokinetic and toxicokinetic studies are presented in table 2. Of the techniques used, we choose the three most widely used sampling techniques to assess the differences in PK parameters using IV and PO route of administration. All oral group rats were dosed through gavage and intravenous animals received the dose through tail vein. The mean pharmacokinetic parameters following single intravenous and oral dose administration of dapsone using different sampling techniques are summarized in table 3.

Following intravenous dose administration of dapsone, the clearance was lower in jugular vein group (3.72 mL/min/kg), followed by retro-orbital group (4.31 mL/min/kg) and saphenous vein group (5.09 mL/min/kg). Overall clearance of dapsone was low in Sprague Dawley rat, less than 10% of liver blood flow of 55 mL/min/kg. The plasma concentrations following intravenous administration declined bi-exponentially with a rapid distribution phase followed by slow elimination phase. Volume of distribution was 2-fold the total body water of 0.7 L/kg confirming that the compound is not highly distributed to the tissues or partitioning into the red blood cells. Dapsone showed high half-life varying between 4.33 h to 5.79 h between groups.

Following oral administration, dapsone absorbed rapidly with T_{max} values ranging from 0.25 h to 4 h. The corresponding C_{max} values were 4600 ng/mL for retro-orbital group, 5540 ng/mL for jugular vein group and 5330 ng/mL for saphenous vein group. The area under the curve (AUC_{inf}) was lower at 38600 ng/mL for retro-orbital group compared to 45400 ng/mL for jugular veinand, 51700 ng/mL for saphenous vein sampling group. The absolute bioavailability of dapsone in retro-orbital sampling, jugular vein sampling and saphenous vein sampling groups was 83%, 84% and 100%, respectively. The exposures obtained in retro-orbital sampling and jugular vein sampling are in agreement with the results published by Helton *et al.*[24]. Mean plasma concentration-time data following intravenous and oral dosing for retro-orbital plexus sampling, jugular vein sampling and saphenous vein sampling are presented in tables 4 to 9 and the corresponding intravenous and oral profiles are shown in figure 1 and 2, respectively.

The exposures (C_{max} and AUC_{last}) following intravenous and oral administration of dapsone in all the groups were similar and were not statistically significant (P value > 0.05).

Discussion

Rats have traditionally been used as a laboratory animal in virtually every area of biomedical research since 1856 including the ADME and PK studies. It has been reported that in rat, certain parameters like plasma glucose, blood coagulation parameters, corticosterone levels, renin activity, serum hormone levels, serum enzyme activities and drug binding to plasma proteins may vary depending on the site and method of blood sampling, the extent of rat handling, acclimatization time and the presence of

Table 1: Study Design for Pharmacokinetic Study.

Sampling technique	Route of administration	Number of animals per group	Dose (mg/kg)	Dose volume (mL/kg)	Formulation strength (mg/mL)
Retro orbital plexus	IV	4	12	3	4
	PO	4			
Jugular vein	IV	4			
	PO	4			
Saphenous vein	IV	4			
	PO	4			

IV: intravenous dosing through tail vein, PO: oral gavage needle

Table 2: Different Sampling Techniques.

Route of sampling	Volume for sampling	Repeated sampling	Anesthesia required	Speed and efficiency of sampling	Sample quality	Sampling stress
Retro-orbital	M-L	Yes	Yes	Slow and need more time	Good	Yes
Jugular vein	L	Very easy	No	Fast and timely	Excellent	No
Saphenous vein	S-M	Yes	No	Fast and timely	Good	Very little
Tail vein	S-M	Yes	No	Fast and timely	Good	Yes
Tail artery	M-L	Yes	No	Fast and timely	Excellent	Yes
Tail clip	S	Yes	No	Fast and timely	Poor	Yes

Adapted from reference: http://oacu.od.nih.gov/ARAC/documents/Rodent_Bleeding.pdf;
S=small, M=medium, L=large

Table 3: Pharmacokinetic Parameters of Dapsone Following Intravenous and Oral Dose Administration.

Group / Sampling Site	Route	T_{max} [a] (h)	C_o/C_{max} [$] (ng/mL)	AUC_{last} (ng.h/mL)	AUC_{inf} [$] (ng.h/mL)	CL (mL/min/kg)	Vss (L/kg)	$T_{1/2}$ (h)	F[b]
I Retro orbital	IV	NA	12100 ± 1640	45700 ± 2530	46500 ± 2390	4.31 ± 0.22	1.35 ± 0.12	4.33 ± 0.17	83
	PO	0.75 (0.25-1.0)	4600 ± 385	37000 ± 2050	38600 ± 627	NA	NA	NA	
II Jugular vein	IV	NA	13400 ± 1150	51700 ± 4700	54300 ± 5410	3.72 ± 0.40	1.50 ± 0.16	5.79 ± 0.97	84
	PO	1.0 (0.5-1.0)	5540 ± 1290	42900 ± 3220	45400 ± 4390	NA	NA	NA	
III Saphenous vein	IV	NA	10700 ± 1880	38400 ± 2360	39400 ± 2330	5.09 ± 0.29	1.64 ± 0.19	4.94 ± 0.49	~100
	PO	2.0 (0.5-4)	5330 ± 1360	49800 ± 11600	51700 ± 12200	NA	NA	NA	

[a]T_{max} reported as median (min-max); [b]AUC_{inf} and nominal doses are used for bioavailability calculation; NA: not applicable; [$]$C_{max}$ and AUC_{inf} of IV and PO groups by all sampling routes found to be statistically insignificant, $p > 0.05$

Table 4: Concentration-time profile of dapsone (12 mg/kg) following tail vein dosing and retro-orbital sampling.

Time point (h)	Plasma concentration (ng/mL)						
	Rat 71	Rat 72	Rat 73	Rat 74	Mean	SD	%CV
0.083	11520	10892	10210	12446	11267	951	8
0.25	9830	9572	10025	9387	9704	281	3
0.5	8533	7447	8918	8920	8454	696	8
1	6934	7085	7387	7187	7148	190	3
2	4862	5854	4899	5154	5192	460	9
4	2590	2561	2505	748	2101	902	43
8	1534	1987	1768	1964	1813	211	12
24	124	104	105	163	124	28	22

Table 5: Concentration-time profile of dapsone (12 mg/kg) following oral dosing and retro-orbital sampling.

Time point (h)	Plasma concentration (ng/mL)						
	Rat 75	Rat 76	Rat 77	Rat 78	Mean	SD	%CV
0	0	0	0	0	0	0	NA
0.25	3600	4976	4515	4647	4434	589	13
0.5	4063	4456	4040	4032	4148	206	5
1	4003	4471	4667	4701	4461	321	7
2	3123	3594	3742	3935	3598	346	10
4	2004	2165	2241	2358	2192	148	7
8	1690	1805	1852	1850	1799	76	4
24	121	149	144	95	128	25	19

NA: not applicable

another previously treated rat. Summary of various parameters in rat used for pharmacokinetic studies are presented in table 10 [10,28].

Blood sampling is one of the most common procedure performed on laboratory animals, and yet there is still a need to refine available techniques both from a welfare point of view and because stressful blood sampling techniques may profoundly affect physiological variables. It has been reported that if animals are handled for more than 5 min, levels of corticosterone increases. The study of compound PK in the rat is solely dependent upon procedures that allow blood sampling. Blood can be sampled from rats in number of ways, for example by puncturing the tail vein, the sublingual vein and the saphenous vein or by implanting vascular catheters. Each method has its advantages and limitations and should be considered in order to minimize the impact of these limitations on experimental results.

Table 6: Concentration-time profile of dapsone (12 mg/kg) following tail vein dosing and jugular vein sampling.

Time point (h)	Plasma concentration (ng/mL)						
	Rat 87	Rat 88	Rat 89	Rat 90	Mean	SD	%CV
0.08	11900	12215	12709	10930	11939	751	6
0.25	8312	9769	10152	9481	9429	793	8
0.5	7349	8138	8011	8325	7956	425	5
1	5682	7028	6649	6823	6546	596	9
2	4054	4980	4257	4941	4558	473	10
4	3021	2843	2787	3636	3072	389	13
8	1704	2205	2380	2276	2141	301	14
24	234	436	204	287	290	103	35

Table 7: Concentration-time profile of dapsone (12 mg/kg) following oral dosing and jugular vein sampling.

Time point (h)	Plasma concentration (ng/mL)						
	Rat 91	Rat 92	Rat 93	Rat 94	Mean	SD	%CV
0	0	0	0	0	0	0	NA
0.25	1276	3559	2005	2214	2263	953	42
0.5	3607	3364	6625	3860	4364	1521	35
1	5213	3855	5844	6452	5341	1112	21
2	3875	3226	3795	4429	3831	492	13
4	3187	2514	2482	2849	2758	331	12
8	1705	2136	2190	2119	2037	224	11
24	265	178	258	422	281	102	36

NA: not applicable

Table 8: Concentration-time profile of dapsone (12 mg/kg) following tail vein dosing and saphenous vein sampling.

Time point (h)	Plasma concentration (ng/mL)						
	Rat 112	Rat 113	Rat 114	Rat 115	Mean	SD	%CV
0.08	8127	11171	9686	10565	9887	1322	13
0.25	8885	9028	8410	8618	8735	275	3
0.5	6419	9106	8656	9272	8363	1322	16
1	6599	6471	3447	4429	5237	1553	30
2	3981	4757	4398	4131	4317	340	8
4	2257	2442	2291	2641	2408	175	7
8	1264	1433	1335	1159	1298	116	9
24	118	129	195	128	143	35	25

Table 9: Concentration-time profile of dapsone (12 mg/kg) following oral dosing and saphenous vein sampling.

Time point (h)	Plasma concentration (ng/mL)						
	Rat 116	Rat 117	Rat 118	Rat 119	Mean	SD	%CV
0	0	0	0	0	0	0	NA
0.25	2393	3734	1417	1870	2354	1003	43
0.5	3504	6538	1854	3419	3829	1959	51
1	4621	5321	2055	4817	4204	1462	35
2	5930	6318	2644	5468	5090	1667	33
4	4027	5496	3397	4783	4426	911	21
8	1747	2739	1795	2313	2149	470	22
24	193	387	232	278	273	84	31

NA: not applicable

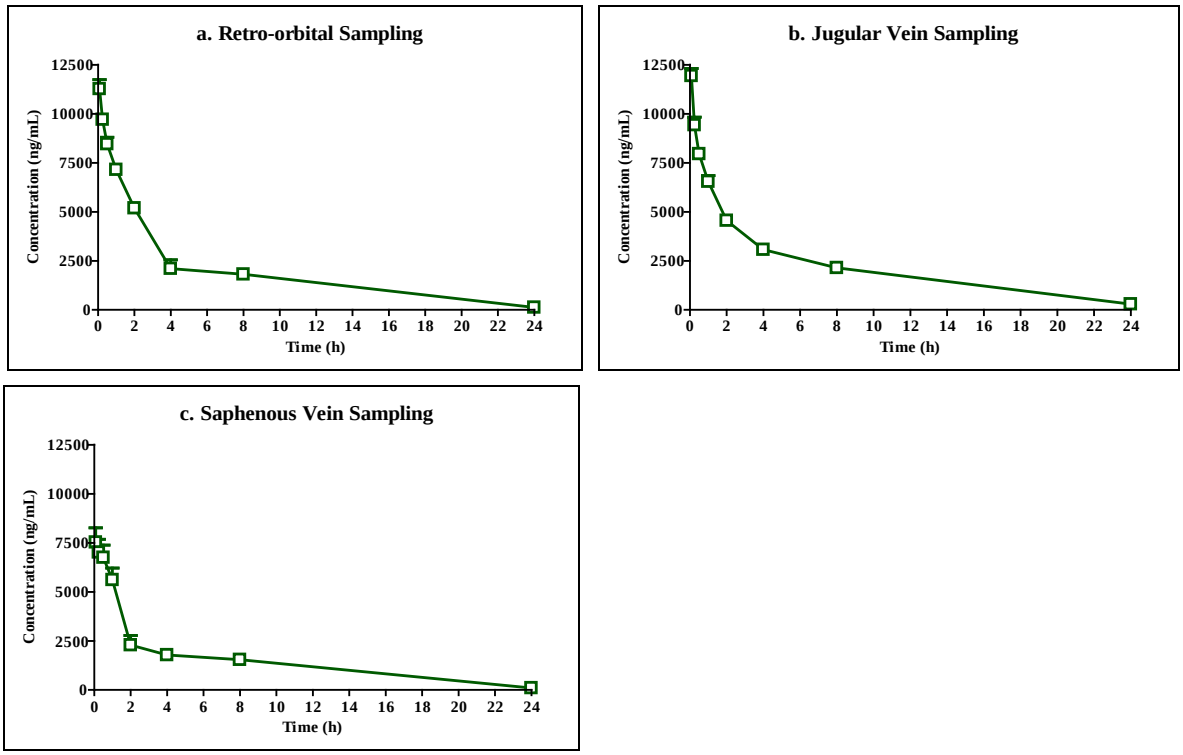

Figure 1: Concentration-Time Profile of Dapsone Following Intravenous Administration (12 mg/kg, n=3, mean + SD).
a. Retro-orbital sampling, b. Jugular vein sampling, c. Saphenous vein sampling

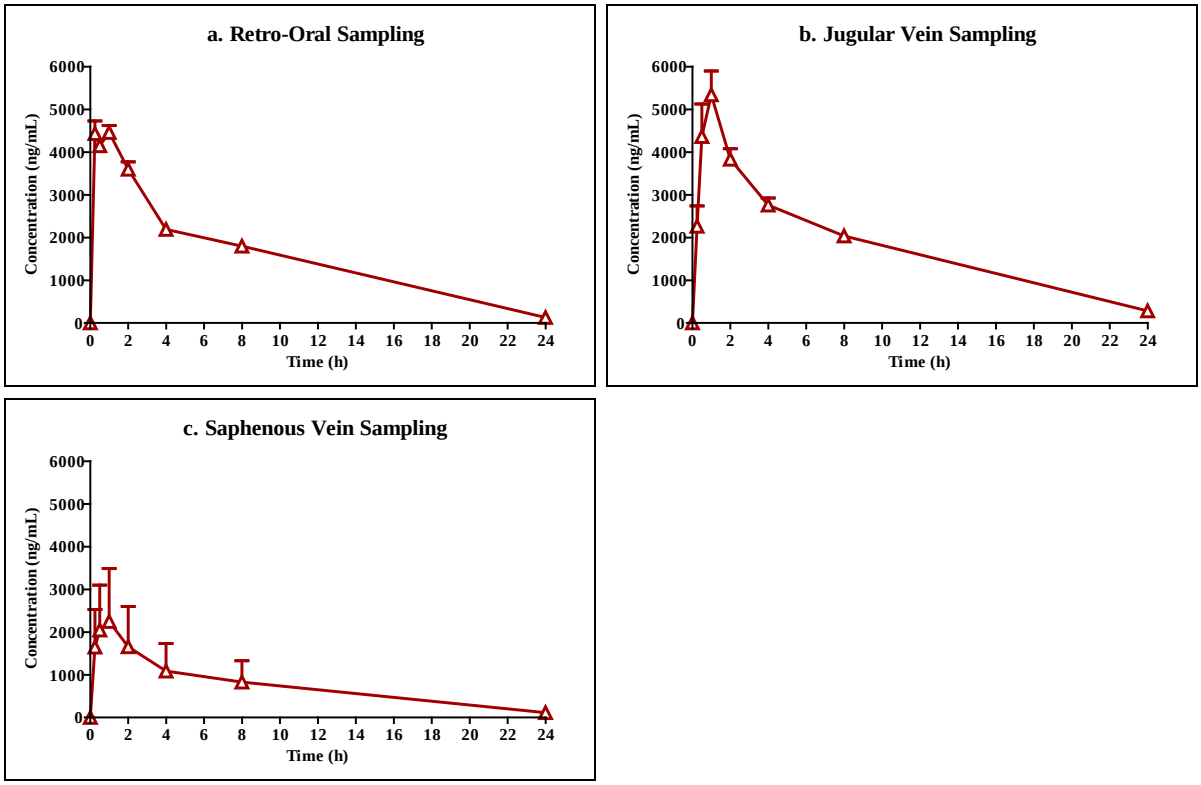

Figure 2: Concentration-Time Profile of Dapsone Following Oral Administration (12 mg/kg, n=3, mean + SD).
a. Retro-orbital sampling, b. Jugular vein sampling, c. Saphenous vein sampling

Table 10: Summary of Various Parameters of Rats used for Pre-clinical Studies[a&b].

Parameter	Range or Mean	Parameter	Range or Mean
Weight	250 g	Birth weight	5-6 g
Life span	2.5-3 y	Heart rate	330-480 beats/min
Surface area	0.03-0.06 cm^2	Plasma protein	6.2 g/100 mL
Plasma Albumin	3.27 g/100 mL	Plasma α-1-AGP	1.25 g/100 mL
Total oxygen consumption	1.59 mL/h/g	Total ventilation	0.025 L/min
Water consumption	80-110 mL/kg/day	Blood pressure	Systolic 88-184 mm Hg Diastolic 58-145 mm Hg
Food consumption	100 g/kg/day	Stroke volume	1.3-2.0 mL/beat
Body temperature	37°C	Cardiac output	50 mL/min
Gestation	21-23 days	RBC volume	3.63 mL/kg
Litter size	8-14 pups	Respiration rate	66-114 per min
Plasma pH	7.4	Urine pH	7.3-8.5
Total body water	167 mL	Intracellular fluid	92.8 mL
Plasma volume	7.8 mL	Extracellular fluid	74.2 mL
Organ weights (g) Brain Liver Kidney Heart Spleen Adrenals Lung	 1.8 10.0 2.0 1.0 0.75 0.05 1.5	Organ volumes (mL) Brain Liver Kidney Heart Spleen Lungs Gut Muscle Adipose Skin Blood	 1.2 19.6 3.7 1.2 1.3 2.1 11.3 245 10.0 40.0 13.5
Blood flow (mL/min) Brain Liver Kidney Heart Spleen Gut Muscle Adipose Skin Hepatic artery Portal vein Cardiac output	 1.3 13.8 9.2 3.9 0.63 7.5 7.5 0.4 5.8 2.0 9.8 74.0	 pH (fed) Stomach - Anterior Stomach – Posterior Small intestine Beginning End Cecum Colon Feces	 5.0 3.8 6.5 7.1 6.8 6.6 6.9
Urine flow GFR	50.0 mL/day 1.31 mL/min	Bile flow	22.5 mL/day
β glucuronidase activity (nmol substrate/h/g contents)	Proximal small intestine - 304 Distal small intestine - 1341	Transit time in small intestine	88 min

[a]Cocchetto *et al.* [10], [b]Davies *et al.* [28]

There are various published reports available where authors have tried to study the impact of site of blood sampling on animal health and compound PK. van Herck *et al.* [17] studied the influence of orbital sinus blood sampling by different technicians on clinical signs in rats and found that experienced animal technicians were able to perform the sampling without causing a statistically significant increase in alterations in punctured orbits. However, the less experienced animal technicians caused severe abnormalities in orbital sinus of rat. The use of either a pasteur pipette or a hematocrit capillary did not produce different results. Neither did puncturing the lateral vs the medial canthus of the orbit. They also reported that by not applying chloramphenicol eye ointment in the conjunctival sac after puncture, the number of abnormalities in ocular discharge and corneal alterations in the punctured orbits were significantly decreased. No statistically significant association was found between the eye position,

ocular discharge, corneal alterations or intra-ocular alterations and the factor number of punctures per orbit.

Orbital sinus blood sampling is a technique used frequently in rats, but it is controversial, particularly due to the ethical and emotional nature attached to it. The BVA/FRAME/RSPCA/UFAW joint working group had stated that orbital puncture is acceptable only as a terminal procedure while the animal is under anesthesia. The behavior of rats after orbital sinus blood sampling under light diethyl ether anesthesia, as performed by a skilled animal technician in the medial canthus of the orbit, was studied previously in an open field, and telemetrically as diurnal locomotor activity and eating pattern. In those studies, the behavior of punctured rats did not differ from that of those treated with only diethyl ether. The clinical conditions of rats after a singular orbital puncture in the medial canthus of the orbit by an experienced animal technician showed no alterations, apart from a possibly higher incidence of enophthalmia in the punctured eyes. vanHerck *et al.* [19] subsequently reported histological changes in the orbital region in rats after retro-orbital puncture. These included haemorrhages and inflammatory reactions in the puncture track, retro-orbital periosteum, eye muscles and Harderian gland.

There are reports that sampling method can influence clinical pathology parameters and renal functional parameters. vanHerck *et al.*[6] studied the effect of retro orbital, saphenous and tail vein bleeding on the behavior and blood parameters of rat. They concluded that the induction of diethyl-ether anesthesia before orbital puncture caused significantly more visible distress than did either the induction of O_2-N_2O halothane anesthesia needed for tail vein puncture or manual fixation combined with saphenous vein puncture. The three blood sampling techniques had no differential effects on the behaviors of grooming, locomotion and inactivity. Of the three methods, orbital puncture appeared to be the fastest technique. It produced the lowest plasma potassium and highest sodium levels, possibly indicating that is caused by lesser erythrocyte damage. The acid-base equilibrium of the blood samples indicated that saphenous and tail vein puncture might have induced a slight alkalosis that might be stress related. Hui *et al.* [26] studied the effect of tail vein, femoral artery cannula and retro orbital sinus bleeding techniques on the pharmacokinetics of six marketed drugs. They recommended tail-bleeding technique and cannulation techniques for pharmacology, toxicology exposure and PK studies, particularly in early discovery work. They concluded that retro-orbital bleeding was controversial and no longer considered a humane method.

For jugular vein sampling, cannulation is performed which typically takes 10 min for an experienced surgeon and is conducted under aseptic condition. The surgicals are autoclaved, sterile gloves are used and after surgery povidone and nesoprine are applied to further prevent infection. After any type of invasive surgery, it is also mandatory to administer analgesics as per the Guide for the Care and Use of Animals. Most widely used analgesic is buprenorphine (which is a controlled substance) and administered at a dose of 0.05 mg/kg through subcutaneous route. Administering buprenorphine although relives pain is associated with side effects like respiratory depression and nausea. In addition, the most critical effect of buprenorphine is on the neural function in the central nervous system leading to consequent changes in behavior. Although buprenorphine clears within 4 h from the systemic circulation in rats, its effects on neural function are long term and there are studies showing relapse of secondary pain after 48-72 h post surgery. Therefore, care should be taken when studying new chemical entities targeted for central nervous system in jugular vein cannulated rats and in general interpretation of PK results for all studied compounds. Care should be taken to ensure that the catheter does not get blocked after surgery and patency should be checked at least once a day. In addition, the personnel have to ensure that the cannula does not come out during the experimental phase, putting the study in jeopardy.

For saphenous vein sampling, the sampling area is shaved and petroleum jelly or silicone oil is applied on the site of sampling to avoid spreading of blood sample. A capillary tube of capacity 50-100 µL coated with anticoagulant is used for sampling, ruling out any coagulation related issues.

Using the three sampling techniques, clearance, volume of distribution and half-life of Dapsone were similar and compared well with the published data. This showed that primary PK parameters were not affected by sampling site. Bioavailability was high and similar in all the groups again confirming no differences due to sampling site. Although we used very well characterized dapsone for assessing the sampling site effect, more compounds needs to be assessed to build a robust data base.

Conclusion

All blood sampling techniques employed are invasive and cause at least some stress and pain if used without suitable anesthesia. The present work compared different sampling techniques like orbital sinus, jugular vein and saphenous vein for determining the PK parameters of dapsone following different intravenous and oral administration. The PK parameters of dapsone were found to be statistically insignificant in spite of using different sampling techniques. Compared to assessed sampling techniques, we found saphenous vein sampling to have the following advantages. For saphenous vein sampling, animals are not catheterized, blood volume drawn can be controlled without hemolysis of samples and no upfront preparation is required to initiate the study at short notice. The only drawback during saphenous vein sampling is the animal handling which may lead to stress. In addition, saphenous vein sampling can be extended to mouse which would not only decrease the animals used in sparse sampling design but would also help to compare the inter-animal variability in PK parameters. Toxicokinetic studies which are typically long term, JVC rats are not preferred as the animals may be prone to infection, cannula may get blocked or cannula may come out during the study.Use of saphenous vein sampling would reduce the number of animals in TK group and at the same time give more robust TK parameters as the samples will be drawn from the same animal. Authors recommended

saphenous vein sampling to be the most appropriate from scientific and ethical perspective for conducting PK and TK studies in rodents. To summarize, SV sampling reduce the number of animals in different PK (mouse) and TK (mouse and rat) studies by using serial draws, offers refinement over the other sampling techniques with minimal preparation upfront and with a potential to replace them.

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