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QUANTIFICATION OF IRINOTECAN (CPT-11) AND ITS METABOLITE, SN-38, IN RAT PLASMA AND BILE SAMPLES: APPLICATION TO PHARMACOKINETIC STUDIES

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INTRODUCTION

Mechanism of Action: Irinotecan is a semisynthetic, water-soluble derivative of camptothecin, which is a cytotoxic alkaloid extracted from plants such as *Camptotheca acuminata*.[3] Irinotecan and its active metabolite, SN-38, inhibit the action of topoisomerase I, an enzyme that produces reversible single-strand breaks in DNA during DNA replication. These single-strand breaks relieve torsional strain and allow DNA replication to proceed. Irinotecan and SN-38 bind to the topoisomerase I-DNA complex and prevent religation of the DNA strand, resulting in double-strand DNA breakage and cell death. The precise contribution of SN-38 to the activity of irinotecan in humans is not known.[4] Irinotecan is cell cycle phase-specific (S-phase).[5]

Pharmacokinetics

Interpatient variability	**high interpatient variability in the pharmacokinetics of irinotecan and SN-38**	
Oral Absorption	rapidly absorbed; no information found on extent of absorption	
	time to peak plasma concentration	within 1-2 h[6]
Distribution	detected in pleural fluid with maximum concentration of 37% for irinotecan and 76% for SN-38 of the corresponding plasma levels[7]; also detected in sweat and saliva.[8]	
	cross blood brain barrier?	no information found
	volume of distribution	125 mg/m^2 dose: 110 □ 48.5 L/m^2; 340 mg/m^2 dose: 234 □ 69.6 L/m^2
	plasma protein binding	irinotecan: 30-68%; SN-38: 95%
Metabolism	primarily hepatic, rapidly converted to SN-38 by hepatic carboxylesterase enzymes; irinotecan and SN-38 undergo reversible, pH-dependent conversion between the active lactone (acidic pH) and inactive hydroxyacid (basic pH) forms.[4]	
	active metabolite	SN-38
	inactive metabolite	SN-38 glucuronide, aminopentane

		carboxylic acid[7]
Excretion	biliary and urinary excretion	
	Bile[9]	25% as irinotecan; 1% as SN-38
	urine	11-20% as irinotecan; < 1% as SN-38
	feces	63.7 - 6.8%[10]
	terminal half life	340 mg/m^2 dose: irinotecan 11.7 - 1.0 h; SN-38 21 - 4.3 h half-life increases with dose but this does not affect the linear relationship between dose and AUC[11]
	clearance	13.3 - 6.1 L/h/m^2
Gender	no clinically significant difference	
Elderly	no clinically significant difference	
Children	greater interpatient variability than in adults, with comparable clearance but shorter half-lives[12,13]	
Ethnicity	no	

USES

Primary uses	***Other uses***
Colorectal cancer[3,14-18]	Cervical cancer[19]
Esophageal cancer[20]	
Gastric cancer[21]	
Glioma[7]	
Lung cancer[22,23]	
Mesothelioma[7]	
Pancreatic cancer[24]	

Special Precautions

Prior radiation: Patients with previous pelvic or abdominal radiation have a greater risk of irinotecan-related toxicities and dose reduction is recommended.[3,19] BCCA protocols recommend irinotecan not be used within six weeks of radiation, especially to the pelvis. Concurrent use of irinotecan as a radiation-sensitizing agent is under investigation.[25]

Pulmonary syndrome: A potentially life-threatening syndrome consisting of dyspnea, fever, and reticulonodular pattern on chest x-ray occurred in some patients with pre-existing lung tumours or nonmalignant pulmonary diseases in early clinical trials. Although the extent to which irinotecan may have been responsible for this complication has not been established, caution is recommended.[3,4]

Hyperbilirubinemia: The risk of severe (grade 3 or 4) neutropenia during the first course of irinotecan therapy may be substantially increased in patients with modest increase in serum

bilirubin (17-35 μmol/L).[3] The use of irinotecan in patients with significant hepatic dysfunction has not been established. In clinical trials, irinotecan was not administered to patients with serum bilirubin > 35 μmol/L, transaminase > 3 times the upper limit of normal (ULN) if o liver metastases, or transaminase > 5 times the ULN with liver metastases.[4]

Gilbert's syndrome: Individuals with Gilbert's syndrome have deficient uridine diphosphate glucuronosyltransferase activity, which is involved in the elimination of SN-38, the active metabolite of irinotecan. Hence, Gilbert's syndrome may increase the risk of irinotecan-induced toxicity.[26] Screening for Gilbert's syndrome using direct/indirect serum bilirubin is recommended.[27] Gilbert's syndrome is characterised by: consistent mild elevation of total serum bilirubin (20-90 μmol/L) indirect (unconjugated) bilirubin should be at least 90% by van den Bergh's test and 99% by high-performance liquid chromatography normal serum ALT and AST hemolysis excluded based on normal hemoglobin, haptoglobin and reticulocyte count.[28]

Carcinogenicity: There is some evidence linking therapy with topoisomerase I inhibitors, such as irinotecan, to the development of acute leukemias associated with specific chromosomal translocations.[4]

Mutagenicity: Irinotecan and its active metabolite SN-38 were not mutagenic in Ames test. However, irinotecan was clastogenic in mammalian *in vitro* and *in vivo* chromosome tests.[4]

ertility: No information found.

Pregnancy: FDA Pregnancy Category D. There is positive evidence of human fetal risk, but the benefits from use in pregnant women may be acceptable despite the risk (eg, if the drug is needed in a life-threatening situation or for a serious disease for which safer drugs cannot be used or are ineffective).[4]

Breastfeeding is.[4]

ORGAN SIGHT	SIDE EFFECT
allergy/immunology	**immunosuppressive**[4]
blood/bone marrow febrile neutropenia	anemia (61%, severe 7%)
	leukopenia (63%, severe 28%)
	neutropenia (54%, severe 26%) 125 mg/m^2 weekly[29]: nadir 15-27 days, recovery 23-35 days 350 mg/m^2 3-weekly[30]: nadir 8-9 days, recovery 19-25 days
	neutropenic fever (3%)
	thrombocytopenia (7%, severe 3%)[30]
	thrombocytopenia, immune (rare)[31]
cardiovascular (arrhythmia)	bradycardia (5%)[32]
cardiovascular (general)	edema (10%, severe 1%)
	hypotension (6%)[32]
constitutional symptoms	chills (14%, severe 0.3%)
	fatigue (76%, severe 12%)
	fever (45%, severe 0.7%)
	sweating (57%)[32]
	weight loss (30%, severe 0.7%)
dermatology/skin	*extravasation hazard*: none[33]
	alopecia (61%)
	flushing (11%, severe 0%)
	piloerection (3%)[32]
	rash (13%, severe 0.7%)
gastrointestinal	*emetogenic potential*: high moderate[29,34]
	abdominal enlargement (10%, severe 0.3%)
	anorexia (55%, severe 6%)
	constipation (30%, severe 2%)
	dehydration (15%, severe 4%)
	diarrhea, early onset (51%, severe 8%); see paragraph following **Side Effects** table
	diarrhea, late onset (88%, severe 31%); see paragraph following **Side Effects** table
	dyspepsia (11%, severe 0%)
	flatulence (12%, severe 0%)
	nausea (86%, severe 17%)
	salivation (11%)[32]
	stomatitis (12%, severe 0.7%)
	vomiting (67%, severe 13%)
hepatic	increased alkaline phosphatase (13%, severe 4%)
	increased AST (11%, severe 1%)
infection	minor infection (15%, severe 0%)
neurology	dizziness (15%, severe 0%)
ocular/visual[32]	lacrimation (12%)
	miosis (10%)
	visual disturbances (15%)
pain	abdominal pain or cramping (57%, severe 16 %)
	back pain (15%, severe 2%)

	headache (17%, severe 0.7%)
	pain (24%, severe 2%)
pulmonary	cough (17%, severe 0.3%)
	dyspnea (22%, severe 4%)
	rhinitis (16%, severe 0%)
secondary malignancy	acute leukemias[4]
syndromes	cholinergic syndrome
	pulmonary syndrome of dyspnea, fever and reticulonodular pattern on chest x-ray

Early onset diarrhea and cholinergic syndrome: Irinotecan can cause both early and late onset diarrhea. Both forms of diarrhea may be severe and appear to be mediated by different mechanisms. Early onset diarrhea occurs during or within 24 hours of administration of irinotecan. It is usually transient and only infrequently severe. Early onset diarrhea is thought to be part of a cholinergic syndrome mediated by increased anticholinesterase activity of the irinotecan parent compound. It may be accompanied by other cholinergic symptoms such as rhinitis, hypersalivation, miosis, lacrimation, diaphoresis, flushing, and abdominal cramping. The cholinergic syndrome is more likely to occur at higher irinotecan dose levels and associated with the onset of peak irinotecan plasma levels.[3] Thus, infusing irinotecan over less than 90 minutes may increase the likelihood of the cholinergic syndrome.[35] Early onset diarrhea and cholinergic symptoms are treated with atropine 0.3 – 0.6 mg IV or SC as needed, repeated up to a maximum dose of 1.2 mg. Blood pressure and heart rate should be monitored during atropine therapy.[27] Prophylactic atropine may be required for subsequent treatments.[30]

Late onset diarrhea: This occurs more than 24 hours after administration of irinotecan and can be prolonged, leading to potentially life-threatening dehydration and electrolyte imbalance.[3] The diarrhea has a median onset of 5 and 11 days after the 3-weekly[32] and weekly[36] dosing schedule of irinotecan, respectively. The median duration of diarrhea for the one-weekly schedule was 3 days, with severe diarrhea (grades 3-4) lasting for 7 days.[3] Late onset diarrhea is thought to be related to abnormal ion transport in the injured intestinal mucosa, leading to increased secretion of water and electrolytes into the intestinal lumen.[37] Management of diarrhea should include prompt treatment with high dose loperamide. Patients with severe diarrhea should be carefully monitored for dehydration and given fluid and electrolyte replacement as needed. Premedication with loperamide prior to irinotecan treatment is not required. However, patients should be instructed to have loperamide on hand and start the following treatment at the first poorly formed or loose stool, or earliest onset of

more frequent bowel movement than usual (NB, loperamide dose used is higher than recommended by the manufacturer): loperamide 4 mg immediately then 2 mg every 2 hours until diarrhea-free for 12 hours may take 4 mg every 4 hours at night[3,38] An alternative regimen of loperamide 4 mg every 3 hours plus diphenhydramine 25 mg every 6 hours has also been used in a limited number of patients.[14] Laxatives may increase the risk of severe diarrhea[3] and patients should be ounselled about laxative use during irinotecan treatment.

***Severe liver enzyme abnormalities*:** Observed in less than 10% of patients, typically in those with known hepatic metastases.

Agent	Effect	Mechanism	Management
anticonvulsants which induce cytochrome P450 (eg, carbamazepine, phenobarbital, phenytoin)[7,39]	may decrease therapeutic and toxic effects of irinotecan	increase irinotecan clearance via multiple mechanisms	may need to increase irinotecan dose for therapeutic effect
bevacizumab[40]	may increase toxic effects of irinotecan	unknown; increases plasma levels of irinotecan active metabolite (SN-38) by 33%	if patient develops severe diarrhea or neutropenia, decrease irinotecan dose as per protocol[41,42]
dexamethasone (chronic dosing)[7]	may decrease therapeutic and toxic effects of irinotecan	increased irinotecan clearance via multiple mechanisms	may need to increase irinotecan dose for therapeutic effect
dexamethasone (antiemetic dosing)[7]	unknown	effect of single-dose dexamethasone on irinotecan clearance is unknown	no clinical interventions appear necessary
docetaxel[43]		no pharmacokinetic interactions observed	
Etoposide[44]	hepatotoxicity	unknown	avoid concurrent use
lopinavir-ritonavir[45,46]	increased toxic effects of irinotecan	decreased clearance of irinotecan metabolites due to CYP3A4 and UGT1A1 inhibition	avoid concurrent use of HIV protease inhibitors with irinotecan
prochlorperazine[3]	increased incidence of akathisia (on weekly schedule)	unknown	avoid on day of irinotecan treatment
St. John's wort[47]	reduced therapeutic and toxic effects of irinotecan	inhibited CYP3A4 metabolism of irinotecan, leading to decreased plasma levels of the active metabolite (SN-38)	avoid concurrent use

Multiple mechanisms have been suggested to contribute to drug interactions. As irinotecan and its active metabolite SN-38 are oxidized by CYP3A4 to two relatively inactive metabolites, most interactions are attributed to inhibition or induction of this enzyme. Coadministration with CYP3A4 inhibitors can potentially lead to significantly increased formation of SN-38 and result in toxicity. Coadministration with CYP3A4 inducers leads to a

reduction in SN-38 plasma levels, which may have a deleterious effect on treatment outcome. Other suggested mechanisms for drug interactions include: induction/inhibition of carboxyl esterase, UGT1A1, and drug transporters.[48]

HIV protease inhibitors. Inhibition of UGT1A1 may interfere with the metabolism of SN-38, an active metabolite of irinotecan, resulting in increased irinotecan-related toxicities. *In vitro* studies in humans have shown that human UGT1A1 was inhibited by several HIV protease inhibitors, with atazanavir exhibiting the greatest inhibitory activity. Additionally, irinotecan is oxidized directly to an inactive metabolite via the CYP3A4 pathway, another pathway inhibited by HIV protease inhibitors. Based on this evidence, it is generally suggested that coadministration of irinotecan and all HIV protease inhibitors should be avoided,[45,46,48] and that in company literature, coadministration of irinotecan with atazanavir is specifically contraindicated.[49]

SUPPLY AND STORAGE

***Injection*:** Pfizer Canada Inc. supplies irinotecan as single use vials (40 mg, 100 mg, and 300mg) in a concentration of 20 mg/mL. Store at room temperature. Protect from light.[48] Novopharm Limited supplies irinotecan as latex-free single use vials (40 mg and 300 mg) in a concentration of 20 mg/mL. Store at room temperature. Protect from light.[50] Hospira Healthcare Corporation supplies irinotecan as single use vials (40 mg, 100 mg and 500 mg) in a concentration of 20 mg/mL. Store at room temperature. Protect from light.[51] Sandoz Canada Inc. supplies irinotecan as latex-free single use vials (40 mg and 100 mg) in a concentration of 20 mg/mL. Store at room temperature. Protect from light.[52]

Parenteral Administration

BCCA administration guideline in *bold, italics*	
Subcutaneous	no information found
Intramuscular	no information found
Direct intravenous	no information found
Intermittent infusion	***in 500 mL D5W over 90 min*** has also been given over 30-60 min[8,19,20]
Continuous infusion	investigational, has been used in clinical trials at lower dosage (12.5 mg/m^2/day) over 96 h[53]
Intraperitoneal	no information found
Intrapleural	no information found
Intrathecal	no information found
Intra-arterial	no information found
Intravesical	no information found

Dosage Guidelines

Refer to protocol by which patient is being treated. Numerous dosing schedules exist and depend on disease, response and concomitant therapy. Guidelines for dosing also include consideration of absolute neutrophil count (ANC). Dosage may be reduced, delayed or discontinued in patients with bone marrow depression due to cytotoxic/radiation therapy or with other toxicities.

Adults:* BCCA usual dose noted in *bold, italics		
Cycle Length		
Intravenous	***3 weeks:***	***350 mg/m²*** (range 200-350 mg/m²) ***IV for one dose on day 1 maximum single dose: 700 mg***[3,4,27]
4 weeks[23]	60 mg/m² IV for one dose on days 1, 8 and 15 (total dose per cycle 180 mg/m²)	
6 weeks	***125 mg/m²*** (range 50-150 mg/m²) ***IV for one dose on days³ 1, 8, 15 and 22*** (***total dose per cycle 500 mg/m²*** [range 200-600 mg/m²]) NB, sometimes referred to as the "weekly schedule	

Dosage in myelosuppression:	modify according to protocol by which patient is being treated; if no guidelines available, refer to Appendix 6 "Dosage Modification for Myelosuppression"	
Dosage in renal failure:	no adjustment required[3,4]	
***Dosage in hepatic failure*:**	3 weeks:	consider lower starting dose for patients with a combined history of prior pelvic/abdominal radiation and modestly elevated total serum bilirubin (17-35 □mol/L)[4]
4 weeks:	consider lower starting dose for patients with a combined history of prior pelvic/abdominal radiation and modestly elevated total bilirubin (17-35 □mol/L)[4]	
6 weeks:	consider lower starting dose (eg, 100 mg/m[2] IV) for patients with a combined history of prior pelvic/abdominal radiation and modestly elevated total bilirubin (17-35 □mol/L)[4]	
irinotecan has not been studied in patients with total bilirubin > 35 □mol/L, transaminase > 3 times the upper limit of normal (ULN) if no liver metastases, or transaminase > 5 times the ULN with liver metastases.[3]		
Dosage in Gilbert's syndrome	3 weeks:	reduce the starting dose[26] to 200 mg/m[2]
4 weeks:	no information found	
6 weeks:	no information found	

***Dosage in diarrhea*[3]**			
NCIC Grade (value)	**During therapy**		**Start of next course**
	4 and 6 week cycles	**4 and 6 week cycles**	**3 week cycle**
1 (2-3 stools/day more*)	maintain dose level	maintain dose level	maintain dose level
2 (4-6 stools/day more*)	reduce by 25 mg/m[2]	maintain dose level	maintain dose level
3 (7-9 stools/day more*)	omit dose, then reduce by 25 mg/m^2 when resolved to grade 2	reduce by 25 mg/m[2]	reduce by 50 mg/m[2]
4 (10 stools/day more*)	omit dose, then reduce by 50 mg/m[2] when resolved to grade 2	reduce by 50 mg/m^2	reduce by 50 mg/m^2

Children:	Irinotecan is currently being studied in children.

MATERIALS AND METHODS

Experimental

Reagent and chemicals

CPT-11(>99%), SN-38(>96) and topotecan (>98%) originated from Dabur Pharma Limited (U.P. India). Disodium ethylene diamine tetra acetic acid was purchased from sigma-Aldrich (stLowis, MO, USA). Acetonitrile of HPLC grade was obtained from J.T. Baker (USA).O-phosphoric acid and DMSO were from Merck Ltd, (India). For pharmacokinetic studies, irinotecan hydrochloride injection (20mg /ml) manufactured by Dabur pharma limited

(U.P.india) was used. Ketamine(Aneket)and xylazine hcl (xylaxin) was obtained from Neon Labs Ltd.(A.P.india), respectively. The water used was of Milli-Q grade purified by a Milli-Q UV purification system (Millipore, Bedford, MA, USA) All other chemicals and reagents were of analytical or HPLC grade as appropriate and procured locally.

HPLC method development

Instrumentation: The liquid chromatographic system LC-2010CHT series (shimadzu, Kyoto, japan) consisting of gradient flow control pump, on –line solvent Degasser, Autosampler, Diode Array Detector and column Oven All the parameters of HPLC were controlled by LC solutions software version 1.21SP1.

Liquid chromatographic conditions: Chromatographic separations were achieved using YMC, C-18, ODS-A RP column(250mm×4.6mm,4um) stainless steel column, which was maintained at ***35º C.*** The sample of 50ul were injected into HPLC system. The mobile phase consisted of 100% acetonitrile (mobile phase A) and Milli Q water, adjusted ph 3.0 with 20% o-phosphoric acid (mobile phase B) at a flow rate of 1.0 ml/min. The column was initially equilibrated at 10%solvent A and 90% solvent B. After injection, the concentration of solvent A was increased linearly to 80% and solvent B decreased linearly to 20% over 15 min. The A:B was then maintained at 80:20 for further 5 min. After 5 min, the system was returned to original conditions (solvent A at10% and solvent B 90%) linearly over 5 min and equilibrated for 5 min before the next injection. The total run time for each sample was 35 min. The mobile phase was filterd through 0.45um filter (sartoriaeratus, Germany) and deaeratd for 15 min by sonication.

Preparation of stock and working standard solution: Primary standard solution for CPT-11 and SN-38 were prepared separately in DMSO at a concentration of 2 mg/ml. A mixture of CPT-11 and SN-38 was obtained at a concentration of 1000ug/ml by diluting the primary stock. Working standard solutions were prepared daily by serial dilution in acetonitrile containing 0.1% glacial acetic acid, to obtain analyte concentrationt from 500ul/mlto 250ng/ml.

Plasma sample preparation: To 100 ul of plasma samples, 100ul ice-cold acetonitrile containing 0.1% glacial acetic acid and 10ul/ml topotecan (as internal standard) was added, resulting in protein precipitation. After rigorous vortex mixing for 5min, the sample was

centrifuged at 3000×g for 10 min at 4º C. An aliquot of 150ul supernatant was transferred to a fresh tube and 50ul was injected onto column for analysis.

Bile sample preparation

Feasibility of various solvent mixtures such as ethyl acetate, dichloromethane (DCM), tetra-butyl methyl ethane (TBME), diethyl ether, n-hexane were evaluated for extraction of analytes from bile. To 100ul of bile sample, 10ul of topotecan (100ug/ml) was added as internal standard. A 1 ml aliquot of DCM: TBME (3:7) was added and the mixture was vortexed, followed by shaking for 10 min (Multireax Heidolph. The labware House, London, UK), subsequently, centrifugation (3000×g for 10 min) was performed to separate the aqueous a. nd organic layer. A 900ul of organic layer was transferred to a clean microtube and evaporated to dryness under vacuum (savant DNA speed –vac, NJ, USA) at 40º C. The dried extracts were redissolved by vortexing and sonication in 150ul of 20:80(v/v) acetonitrile : water (pH3.0) A 50ul volume was injected onto the HPLC system.

Validation procedure: The chromatographic method was further validated for specificity, linearity, sensivity, precision and accuracy according to ICH guideline (12).

Linearity

All validation runs were performed in triplicates on three consecutive days to assess inter-day variation. Seven point calibration curves were constructed in plasma and bile for the two analytes over the selected concentration range and tested for linearity. Plasma calibration standards were prepared at25, 50, 100, 500, 1000, 5000 and 10,000ng/ml whereas, bile calibration standard were 100, 50, 20, 10 2, 1, and 0.5 ug/ml.

Specificity, sensitivity, accuracy and precision

Specificity of the method was determined by analyzing six different batches of blank plasma or bile obtained from healthy rats. The reproducibility of the analytical procedure was determined by calculating intra-and inter-day coefficient of variation (C.V.). Accuracy was performed by five replicate analysis of spiked quality control samples at three concentrations using plasma (500, 100, and 50 ng/ml) and bile (50, 10 and 1 ug/ml) followed by their comparison with the calibration curves prepared on the same day and on different days. Limit of qualification (LOQ) is a lowest concentration at which precision expressed by % C.V. is less than 20% and accuracy expressed by relative difference of measured and nominal is also

lower than 20%. Limit of detection (LOD) was determined at lowest concentration to be detected, taking into consideration a signal-to-baseline noise ratio of 3.

Extration efficiency

The extraction efficiency from plasma and bile was determined by comparison between areas of pure unextracted standards in the relevant concentration ranges plasma (500, 100, and 50 ng/ml) or bile (50, 10, and 1 ug/ml) prepared in mobile phase, with those of extracted biological concentration containing the same amount of drug.

Application of pharmacokinetic studies

Healthy female wistar rats (180-200g) used for pharmacokinetic studies were obtained from breeding stock of Dabur research foundation (Ghaziabad, U.P., india). All animal procedures were approved by institutional Animal Ethics committee (Dabur research Foundation, U.P., india). Rats were fasted over night before dose administration and approximately 3 h post-dose. The target dose was administered intravenous via the lateral tail vein (20mg/kg) or orally via gavage (80mg/kg) using a ball-tipped needle. Blood sample were withdrawn prior to dosing and at various time points till 24 h post-dosing from retro-orbital plexus into microtubes containing disodium ethylene diamine tetra acetic acid, to determine levels of CPT-11 and SN-38. Plasma was obtained by immediate centrifugation at1500×g for 10 min at 4º C. Bile cannulation experiments were carried out as described (11). Briefly, the abdominal cavity was opened after induction of anesthesia with ketamine (90mg/kg i.p.) and xylazine (10mg/kg i.p.) The common bile duct was ligated and cannulated with PE10 polyethylene tubing (inner diameter, 0.28mm; outer diameter, 0.61mm; Harvard apparatus, Holliston MA USA) During the experimental period body temperature of the rats was maintained aat 37˚C with a heating pad. Bile (1ml at each time interval) was collected at 0-1, 1-2, 2-3, 3-4, and at a 5 h, the were sacrificed. The plasma CPT-11 and SN-38 concentration-versus-time curves were used to determine maximum plasma concentration (C_{max}), to maximum plasma concentration (t_{max}), area under the time concentration time curve to the final sampling point ($AUC_{0\text{-}last}$), volume of distribution (V_{ssobs}), elimination rate constant (K_{el}),half life (t1/2) and total body clearance (C lobs). C_0 was the initial plasma Concentration of drug obtained by back-extrapolation to y-axis. Pharmacokinetic parameters were calculated by non compartment model using WinNon –Lin 5.0 programme (Pharsight, Mountain view, CA, USA,). The absolute bioavailability (F) of CPT-11 after the oral

administration (80mg/kg) compared to intravenous (i.v.) administration (20mg/kg) was calculated as follows:

$$F = \frac{AUC_{oral} \times \text{i.v. dose}}{AUC_{i.v.} \times \text{oral dose}}$$

Techniques of blood collection

Total Blood Volume and Recommended

Maximum Blood Sample Size

The total volume of blood that can be safely collected from small exotic mammals for blood analysis should be based on the size of the animal being bled. A standard of collecting 1% of the animal's lean body weight is often given as the best estimate for a safe sample volume; however, there are a number of cases where sampling at this volume is not recommended. For example, it is generally recommended that smaller volumes of blood be collected for patients that are geriatric or suspected of being anemic or hypoproteinemic. It is important for the veterinarian to consider the potential negative effects of collecting too much blood from a compromised or obese patient. Ideally, the amount of blood collected from a patient should be based on published data of the total blood volume of a given species. However, before using the published figures, one needs to know the methodology used in determining this data. Depending on the methodology used, published calculations of total blood volume can vary up to 10%. The most accurate techniques use sodium radiochromate or sodium pertechtate as a red cell label to determine red cell volume, and human serum labeled with radioiodine as a plasma label to measure plasma volume.14 Table 1 gives published figures on whole blood volumes and whole blood volume as percentage of body weight; unfortunately, the methodology used to calculate these data is not described. There are other physiologic factors that can affect the volume of blood that can be safely collected from a patient. For example, as certain species of animals increase in size, their percentage of blood volume decreases (Table 2); therefore, a range of percentages is required for accurate interpretation.14 In addition, there can be differences in total blood volume when comparing different species and age of the animal. The most accurate estimates of blood volume are made based on lean body mass or surface area, but performing these calculations in a busy veterinary hospital is impractical.14 It is best for the veterinarian to err on the side of caution by using a lower blood sampling volume estimate (0.8%) of lean body mass and collecting that volume no more than once every 14 days. Table 3 compares the sample volume that can

be collected when limited to 0.08%, 1%, or 10% of the animal's body weight for some small exotic mammals.

Blood Collection Procedures

Although it can be difficult to collect blood from the small veins of exotic mammals, one can take some consolation in knowing that there are similarities in the vascular anatomy between these animals and larger domestic species that allow for similar approaches for collecting blood samples. Some of the difficulties encountered with exotic mammal venipuncture can be attributed to anatomy, such as a short neck or tail.

Animals that are not used to being handled, or which are otherwise difficult to restrain, should be anesthetized for physical examination and blood collection. For any species that the veterinarian is unfamiliar with, isoflurane anesthesia should be considered. However, isoflurane can adversely affect the results of blood parameters in some exotic small mammals (e.g., ferrets), and these limitations must be considered when interpreting the results.[16,17]

Blood collection on an alert animal can avoid the potential effects that anesthetic agents have on the laboratory test results. Ideally, animals should be fasted for several hours before collecting blood, in the event anesthesia is needed for blood collection or collection of samples for other diagnostic procedures and also to minimize the effect that a recent meal may have on test results. One must also consider the medical problems that the animal presents with and risks that fasting may have on the animal's health. To maximize the recovery of a sample, blood can be collected from the hub of the needle. Before collecting a blood sample, heparin can be drawn up in the needle and the excess expelled from the hub of the needle. Because anticoagulants can affect red blood cell morphology, it is also important that some blood smears be prepared without anticoagulants at the time of blood collection. Laboratory results may vary depending on whether serum or plasma is submitted for analysis. One should always verify with the diagnostic laboratory which sample type will yield the most valid test result. The plasma to blood cell ratio should be estimated at 1:1, but the yield of plasma from a blood collection may only be up to one third of the total samplecollected depending on the physiologic state of the patient. In small exotic mammals, restoration of blood volume after sample collection usually occurs within 24 hours; however, it could take longer depending on the life span of the red blood cells being examined.

In some cases it may take up to 2 weeks or longer for the hemoglobin, hematocrit, and total red blood cell count to return to normal. If blood loss exceeds 20% to 25% of total blood volume, it can lead to hypovolemic shock and death if not treated immediately. It is, therefore, imperative that hemostasis after blood collection is confirmed and that staff are familiar with the signs associated with stress, anemia, and shock so that immediate treatment can take place if any of the critical signs are noted (Table 4).19 Many of the smaller exotic mammals have veins too small to bleed with a needle and syringe. It may be necessary to punch or lance the blood vessel, or, if the vein cannot be seen or felt, it may need to be accessed using certain landmarks and doing a "blind stick." The skin over the site should be aseptically prepared by cleaning with a chlorhexidine gluconate solution or scrub (ChlorhexiDerm Disinfectant Solution or ChlorhexiDerm Plus Scrub; DVM Pharmaceuticals, Inc., Miami, FL USA) or 70% alcohol (isopropyl alcohol 70%; Veterinary Products Laboratories, Phoenix, AZ USA) followed by the application of a topical anesthetic cream (lidocaine 2.5% and prilocaine 2.5%, EMLA cream; AstraZeneca LP, Wilmington, DE USA) over the vein 30 minutes before the blood collection procedure. Restraint tubes can be used for several of the small exotic mammals, and it is important to wash and disinfect these devices between sessions to remove pheromones and to minimize the spread of disease. If the animal that is being restrained has an infection or is stressed by being in the tube, the patient may release pheromones, which can affect the next animal placed in the restraint device. Only an appropriate-sized tube for the individual animal should be used. Monitoring of the patient is necessary to prevent hyperthermia or the development of respiratory compromise due to malposition within the restraint tube.

Venipuncture Techniques in Exotic

Small Mammals

Mice (*Mus musculus*)

To circumvent their small size and short neck, a number of anatomic sites have been identified to collect blood samples in mice. Most of these sites have been developed in research settings where many mice are bled one right after another. With training, staff can quickly become proficient in accomplishing difficult blood-collecting procedures and minimize the stress on the mouse. Some of these collection sites may not be routinely suited for veterinary hospitals but are included here as a possible technique that can be used in an emergency. It is highly recommended that isoflurane anesthesia be used for blood collection for the mouse patient, a species very prone to being stressed by transport, restraint, and being

moved into a new cage. In one study, transportation caused increased corticosterone levels in mice for 48 hours,1 whereas in another study, the corticosterone levels rose after a brief 12-minute transport and the white blood cell count significantly decreased at 4 hours and returned to normal only 12 hours after transport. If there is a problem obtaining blood from one site, other sites may then be quickly accessed without prolonging physical restraint and thus further stressing the mouse and affecting the laboratory test results. If possible, the mouse should be anesthetized in its home cage and allowed to recover in the same cage. By consistently using anesthesia for blood collection, the effects that the anesthetic has on the blood test results will be "standardized" across all of the individual mice sampled, rendering in-house blood reference ranges consistent for comparison.

Lateral Saphenous Vein.

Increasing a mouse's body temperature will help to dilate blood vessels before bleeding.22 Warming the mouse can be accomplished by placing the animal's cage 6 to 8 inches under a low-watt (100-W) light bulb, placing the cage on a heating pad set at a low setting, or placing the cage in an incubator at 102°F (39°C) for 5 to 10 minutes. The mouse should be observed frequently while being warmed because overheating will affect blood test results and can lead to dehydration and hyperthermia. The incubator should be calibrated frequently and monitored for any hot spots. A mouse can either be anesthetized with isoflurane or manually restrained for blood collection. If general anesthesia is not used, a topical anesthetic cream (e.g., EMLA cream) should be applied over the vein 30 minutes before blood sampling. Commercially available restraint tubes are produced for research and can also be used to restrict the movement of an animal for venipuncture for mouse restraint devices are an appropriate-sized plastic centrifuge tube or syringe case (35 mL) that has 5 holes punched or drilled in the end for ventilation. The tube should be rendered opaque or covered in the area of the animal's head to calm the animal. Masking tape is normally placed over the open end to partially cover the tube and allow the rear leg to be pulled out while keeping the animal in place. To prevent the sticky side of the tape from contacting the mouse's fur on the inside of the tube, an additional square piece of masking tape should be used to cover the adhesive at this location. For optimum exposure of the vein, the fur on the caudal surface of the mouse's thigh is clipped and the skin cleaned with alcohol. Petroleum jelly (Vaseline; Unilever United States, Inc., Englewood Cliffs, NJ USA) or silicone grease (McNett Silicone Grease 100% Pure Silicone Lubricant; McNett Corp., Bellingham, WA USA) is then applied over the vein to prevent the blood from spreading away from the site into the fur and thus aid in pooling the

blood for easier collection. A tourniquet (e.g., rubber band clamped with a hemostat) can be placed just proximal to the stifle, and gentle pressure and tension can be applied at the same site to stretch the skin over the hock and stabilize and dilate the vein. Instead of using a tourniquet, the fold of skin between the tail and thigh can be grasped between the thumb and the index finger to extend the leg and hold off the saphenous vein. The lateral saphenous vein can be entered or pricked with a 22- to 30-gauge needle and the pooled blood collected with a microhematocrit tube (BD Plastic Clad Microhematocrit Tubes; Becton, Dickinson and Company, Franklin Lakes, NJ USA).22 Up to 0.2 to 0.3 mL of blood can be safely collected from mice. The foot and leg are then flexed and/or dry gauze is used to supply slight pressure to aid in hemostasis. Later, additional samples can be collected by removing the blood clot or scab from the area. joint, the orbital and submandibular veins join to form the jugular vein. Where these vessels meet is a site from which one can collect 0.2 to 0.5 mL of blood using either general anesthesia or manual restraint (Fig 1). If manual restraint is performed, the mouse should be scruffed with the thumb and index finger to stabilize the submandibular vein while the little finger holds the base of the tail to the palm of the hand. An 18- to 23-gauge needle, #11 scalpel blade, or a mouse-bleeding lancet (Goldenrod Animal Lancet; MEDIpoint Inc., Mineola, NY USA) can be used to puncture the skin over the vessel at a 90° angle to the skin.23 The lancet, needle, or blade must be quickly removed to collect the maximum amount of blood. Blood can be collected with either a microhematocrit or Microtainer tube (BD Microtainer Blood Collection Tubes; Becton, Dickinson and Company). Once the blood sample is collected, the tension on the skin of the neck can be relaxed slightly and light pressure with dry gauze can be applied to the punctured area for hemostasis. Several samples can be collected in one day using this method by removing the scab or blood clot or lancing the vessels on the other cheek. The potential risks associated with collecting samples with this method include inadequate penetration (i.e., too shallow or too deep), lacerating the skin and causing tissue damage, compromising the buccal surface and hereby causing bleeding into the mouth, or lancing too high above the vein resulting in bleeding into the ear canal.

Jugular Vein.

The jugular vein can be used to collect blood from an adult mouse, but success may be limited because of the animal's size. For jugular venipuncture, the mouse should be anesthetized with isoflurane and held in the palm of the nondominant hand. A loop of string from a dry gauze square can be looped around the upper incisors so that the head can be pulled dorsally with the gauze. The ventral surface of the animal's neck should be shaved and

the site disinfected and dampened with alcohol. The jugular veins will appear as blue lines running from 2 to 4 mm lateral to the junction of the sternum and the clavicle up to the angle of the jaw. Blood is collected with a 25-gauge needle attached to a 1-mL syringe or a 25-gauge needle alone. It helps to bend the needle at the hub about 30° to better access the vessel. If the jugular vein cannot be visualized, insert the needle 2 to 4 mm lateral to the sternoclavicular junction at a depth of 1 to 3 mm in the direction of the angle of the jaw. Stabilization is achieved by passing the needle through muscle. Up to 0.2 to 0.3 mL of blood can be collected safely from a mouse jugular vein. Gentle pressure with dry gauze should be applied over the venipuncture site for hemostasis. The anesthetized mouse can also be placed on a sloped, rectangular restraint bleeding board. The board is folded in the middle on the long axis, which creates an angle of 30°. At each end of the fold are 2 pieces of string used to tie the mouse's forelimbs. The anesthetized mouse should be placed in dorsal recumbency with its back lying parallel to the long axis of the board, and with its neck lying on the fold so that its head can be extended dorsally. The mouse's front legs should be secured with the string and tied out laterally from the body. The body and the rear legs should be supported by an assistant so the mouse does not slip down the sloped board. The animal's breathing should be monitored. The fur should be clipped over the neck and the site prepared aseptically. The procedure for collecting the sample from the jugular.

Lateral Tail Vein.

The mouse should be anesthetized for the procedure and placed in lateral recumbency. The lateral tail veins are observed on the lateral aspect of the tail at its base. The veins will need to be dilated with the techniques described for the saphenous vein or by placing the tail in warm water (95-104°F, 35-40°C) for 5 to 10 seconds. The disadvantage of using the tail vein is that once entered, it stimulates the sympathetic nervous system, causing vasoconstriction of the vessel.25 To obtain access to the tail vein, the vein should be held off at the base of the tail. This can be accomplished with a tourniquet designed by Minasian, which can be made from a 2-, 5-, or 10-mL syringe and 20 to 30 cm of 2-0, 3-0, or 4-0 silk, linen, or nylon suture.26 To make the tourniquet, remove the plunger from the syringe and pass a needle and thread through the injection port of the syringe, leaving 1 to 2 inches of the thread hanging out.

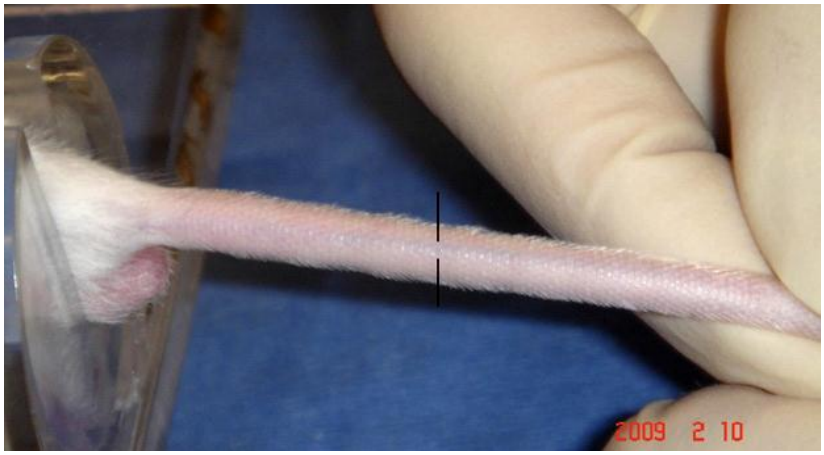


Figure. 2. Mouse. The dilated lateral tail vein is identified between the two black lines.

The needle and thread are then passed through the top of the barrel and passed through the rubber plunger, perpendicular to the shaft of the plunger. The needle and thread are then inserted back down the barrel and out the injection port. The thread is cut off or pulled out of the needle and the two ends are tied together. The knot is then pulled around and back into the injection port and barrel until it rests against the plunger. Next, the plunger can be placed back into the barrel, all the while keeping a loop of thread hanging out of the injection port of the syringe. This loop can then be put around the base of the tail and tightened down by withdrawing the plunger; pushing the plunger down will loosen the tourniquet. Adjustments to the thread length will need to be made based on both the size of the syringe and the desired loop size (Fig 3).26 The tail should be aseptically prepared for sample collection. Alcohol can be used to disinfect the tail, and it should be applied one third to one half of the distance from the tail tip. Holding the tail at this site with the thumb and index finger of one's nondominant hand will stabilize the section of tail from which blood is to be collected. A 25- to 27-gauge needle alone or attached to a 0.5- to 1-mL syringe is inserted into the skin parallel to the vein and then passed into the vein for 2 to 3 mm (Fig 4). The plunger is withdrawn very gently to reduce the chance of venous collapse. A 25-gauge butterfly needle, with the tubing cut off 2 to 5 mm from the hub of the needle can also be used, with the blood being allowed to drip into a microtainer. If there is a problem collecting the initial sample, another attempt, 0.5 cm cranial to the original site, may be made. Up to 50 _L to 0.2 mL can be safely collected from the mouse tail vein. Hemostasis is performed with light pressure over the site with dry gauze as described above. The lateral tail vein should not be squeezed or milked, because this technique can result in a leukocytosis by mixing the marginated white blood cells with the sample or can contaminate the sample with tissue fluids. Historically, collecting a blood

sample from the lateral tail vein has been performed on nonanesthetized animals with a needle or a scalpel blade to cut the vein and then collect the blood into a hematocrit tube or microtainer. This laboratory technique should not be attempted on mice that present to a veterinary hospital. If this procedure is ever used, a topical anesthetic cream should be applied to the tail 30 minutes before the blood sample is collected. The animal should be placed into a restraint tube as described for the saphenous vein and the tail pulled out instead of the rear leg. Lacerating the lateral tail vein will likely produce a sample that is a mixture of tissue fluid and venous or venous blood.

Ventral Tail Artery: Blood collection from the ventral tail artery is similar to that described for the lateral tail vein, except that the vessel being used is the single ventral tail artery. The tail should be warmed as described above to increase blood flow. Up to 0.5 to 0.75 mL of blood can be collected from the tail artery. One should avoid squeezing or milking the tail during sampling, because it may collapse the artery and slow or impede blood flow. In addition, a blood sample collected from a tail that has been squeezed is more likely to contain a mixture of tissue fluids and arterial and venous blood. Direct pressure can be applied to achieve hemostasis, although pressure will need to be applied for a longer period of time because the sample site is an artery. General anesthesia should be used for blood collection from the ventral tail artery; however, if there are concerns regarding the risk of anesthesia to the patient, a modification of this technique may be used that does not require a restraint tube or tail warming. Thirty minutes before the procedure, the site for sample collection should be cleaned and a topical anesthetic cream (e.g., EMLA cream) applied. The mouse should be placed on a smooth table and nondominant hand and the last finger. The mouse's tail should be held with the thumb on the dorsal part of the tail and the index finger on the ventral aspect. The tail is pulled straight up perpendicular to the table. The base of the tail can be stabilized by placing the second and third fingers below the thumb on the dorsal surface of the tail. A scalpel blade is then used to cut across the ventral tail artery at a slight oblique angle to the vessel midway down the length of the tail. Blood can be collected in a microtainer or microhematocrit tube, after which light pressure is applied to the incision to control bleeding. If subsequent samples are needed, they can be collected more proximately on the tail. In one study, 5 blood samples were collected for plasma corticosterone levels by this method every 2 to 3 days for up to 9 days, and the plasma corticosterone levels did not significantly vary over the sampling period. Histological changes at the sampling sites showed minimal inflammation and rapid healing.

Orbital Sinus

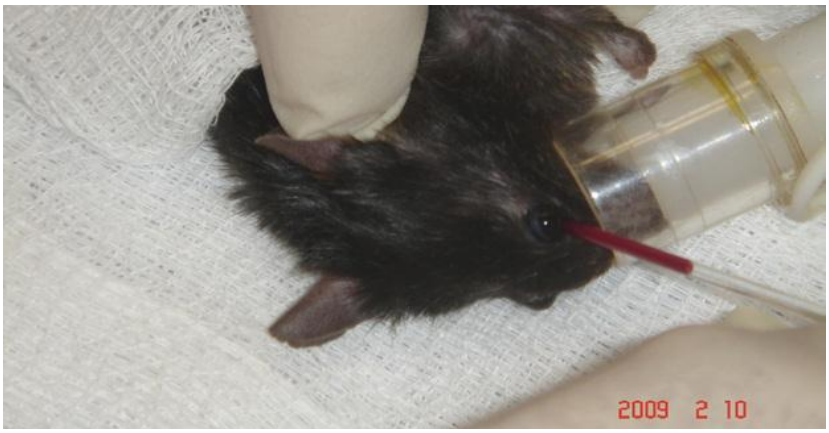


Figure. ***Orbital sinus bleeding in a mouse. A microhematocrit tube is inserted in the medial canthus of the eye. The microhematocrit tube is rotated while applying gentle downward pressure, and the tip is directed toward the middle of the eye socket by directing the tip at a 30° to 45° angle to the side of the head. Once the blood is seen in the tube, it should be withdrawn slightly to facilitate filling.***

There are numerous associated health risks when blood is collected from the orbital sinus of a mouse, which increase if the animal is not anesthetized. The health risks include orbital bleeding with increased pressure on the back of the eye and associated pain, infection, blindness, corneal ulceration, punctured or ruptured globe, keratitis, pannus formation, microphthalmia, proptosis of the globe, panophthalmitis, and fractures of the orbital bones. When this venipuncture method is performed by highly skilled technicians in a research setting, it can be done with few complications and minimal stress to the animal; however, it is rare nowadays for research facilities to use the orbital sinus to collect blood from mice. Anesthesia is recommended when collecting blood from the orbital sinus. A drop of topical ophthalmic anesthetic solution (Proparacaine Hydrochloride Ophthalmic Solution, 0.5% USP; Bausch & Lomb, Inc., Tampa, FL USA; Tetracaine Hydrochloride Ophthalmic Solution USP, 0.5%; Bausch & Lomb, Inc.) should be applied to the surface of the eye and any excess removed after 5 to 10 seconds with dry gauze or a cotton swab. The animal should be placed in lateral recumbency on a table or held in the palm of the nondominant hand so that its head is pointing downward. The index finger should be placed above the eye and the thumb below the eye to pull the skin away from around the globe. This activity will cause the globe to protrude. While restraining a mouse for this procedure, special care must be taken not to occlude the trachea. Using a microhematocrit tube or a fine-walled (1-2 mm outside diameter) borosilicate glass Pasteur pipette, insert the tip in the corner of the eye socket at the

medial or lateral canthus. The tip should be directed toward the middle of the eye socket by directing the tip at a 30° to 45° angle to the side of the head. The tube should be rotated while applying gentle downward pressure until blood is seen in the tube. Once blood is observed in the tube, slightly withdrawing it will increase the blood flow from the sinus. Once the blood stops flowing, the tube is removed and the eyelids are pulled together and pressure is applied to the globe. The skin around the eye should be wiped with dry gauze to remove any blood, being careful not to touch the cornea. No ophthalmic ointment should be applied, because it may cause the animal to rub its eye. The mouse should be monitored for 30 minutes for swelling and/or bleeding from the collection site. Up to 0.2 to 0.3 mL of blood can be safely collected from this site, but it is important to recognize that the sample is a mixture of blood and tissue fluid. Before use, the microhematocrit tube should be checked for any rough edges that could increase tissue injury around the globe. When using the Pasteur pipette, one should cover the open end of the pipette with a finger before removing it from behind the eyeball to prevent blood from dripping out. If blood is collected from the orbital sinus, at least 21 days are required between bleedings from the same eye. Blood collection should be alternated between the two eyes and done no more than twice on each eye.

Tail Clipping, Tail Cut Bleeding.

These aggressive bleeding techniques should be done with the animal under general anesthesia and only if all other sites are unavailable. It is generally recommended that these techniques only be used for terminal patients because of the associated postrecovery pain. The tail tip is prepared aseptically and a topical anesthetic cream (e.g., EMLA cream) is applied 30 minutes before blood collection. The tail should be warmed as previously described to facilitate vasodilatation. A tourniquet is placed around the tail base, and the distal 1 to 2 mm of the tail is amputated perpendicular to the axis of the tail with a scalpel blade or sharp scissors. Approximately 10 _L of blood can be collected in a capillary tube. The blood collected is a mixture of venous, arterial, and tissue fluids. The collection site should be held off with dry gauze for 30 to 45 seconds. Only the fleshy part of the tail tip should be cut and not any skeletal structures (e.g., caudal vertebrae and/or anterior cartilage). If no blood is observed when the first cut is made, another cut should be made 2 to 3 mm proximal to the initial attempt. For a serial blood sample collection, the scab or blood clot can be removed as needed, with no more than 4 samples being collected over a 24- hour period.

Dorsal Pedal Vein, Lateral Marginal Veins of the Tarsus.

The mouse should be placed under general anesthesia for these blood collection techniques. The mouse is warmed to increase its body temperature. The hind foot is held at the ankle by placing the thumb on the top of the foot and the index finger on the plantar aspect. The area is aseptically cleaned and petroleum jelly or silicone grease is applied over the vessel. A 25- to 27-gauge needle is placed in the vein and blood is collected from the hub of the needle in a microhematocrit tube, or the vein is nicked with a 25- to 27-gauge needle and blood is collected in a microhematocrit tube. If the latter technique is used, then a topical anesthetic cream (e.g., EMLA cream) should be applied to the foot 30 minutes before the procedure. Hemostasis can be achieved by applying direct pressure with dry gauze to the venipuncture site. If general anesthesia is not available for the procedure, the animal can be placed in a restraint tube and sampled with the technique described for the saphenous vein. The mouse may show signs of lameness after this blood collection procedure.

Venipuncture Sites for Terminal Blood Collection

Axillary Region.

For blood collection from the axillary region, general anesthesia must be used. Once anesthetized, the mouse should be placed in dorsal recumbency with the front leg abducted laterally. A scalpel blade or sharp scissors should be used to incise the skin over the axillary region. The skin at the caudal edge of the incision should be lifted with a forceps to form a pocket. The axillary vessels should be incised and the blood, up to 0.8 mL, collected with a microhematocrit tube or microtainer. The blood sample collected from the axillary area will contain a mixture of venous and arterial blood and tissue fluids. After collecting blood from the mouse, it should be humanely euthanized.

Laparotomy or Thoracotomy Sites.

After placing a mouse under general anesthesia, one can collect blood from the posterior vena cava through a laparotomy incision or from the aorta or heart via a thoracotomy. The laparotomy procedure is initiated by incising the skin 1 cm caudal to the rib cage. Once in the body cavity, the widest part of the posterior vena cava can be located between the kidneys after displacing the intestines to the left and the liver in a cranial direction. Blood is collected with a 23- to 25-gauge needle attached to a 1-mL syringe. Up to 0.8 mL of blood can be collected, after which the mouse should be humanely euthanized. Blood can also be collected from the aortic arch and the heart via a thoracotomy.

Cardiocentesis: Cardiocentesis for blood collection is a terminal procedure only and must be done with the animal under general anesthesia. The mouse should be placed in dorsal recumbency for the procedure. A 22-gauge, 0.02- to 0.04-inch (0.5-0.9 mm) needle on a 1-mL syringe can be used for the procedure. The needle should be inserted slightly left of midline under the xiphoid cartilage at a 20° to 30° angle from the horizontal axis created by the sternum. While advancing the needle toward the heart, apply slight negative pressure until blood enters the hub to confirm that the heart has been penetrated. Another position from which cardiocentesis can be achieved is by holding the mouse vertically by the fur on the nape of the neck. A 22-gauge, 1-inch needle can be inserted 0.5 cm cranial to the center of the thorax and directed at a 25° to 30° angle cranially. Blood will be seen in the hub of the needle after the needle penetrates approximately 5 to 10 mm into the thoracic cavity. A third method for cardiocentesis Blood Collection in Small Mammals**.**

Blood Plasma Sample Collection and Handling for Proteomics Analysis

A guide to obtain optimal plasma samples

1 Introduction: Blood can be regarded as a complex liquid tissue that comprises cells and extra cellular fluid. The choice of a suitable specimen collection protocol is crucial to minimize artificial processes (e.g. cell lysis, proteolysis) occurring during specimen collection and preparation. Preanalytic procedures can alter the analysis of blood-derived samples. These procedures comprise the processes prior to the actual analysis of the sample and include steps needed to obtain the primary sample (e.g. blood), and to obtain the analytical specimen (e.g. plasma, serum, cells). It has been reported that the most frequent faults in the preanalytical phase are the result of erroneous procedures for sample collection (e.g. blood drawing from an infusive line resulting in sample dilution). The design of blood collection devices may aid in correct sampling: evacuated containers sustain the draw of the accurate quantity of blood to ensure the correct concentration of additives or the correct dilution of the blood, such as in the case of citrated plasma. The speed of the blood draw is also controlled and restricts the mechanical stress.

The favoured site of collection is the median cubital vein, which is generally easily found and accessed. As such, it will be most comfortable for the patient and should not evoke additional stress. Preparation of the collection site includes proper cleaning of the skin with an alcohol (2-propanol). The alcohol must be allowed to evaporate, since commingling of remaining alcohol with the blood sample may result in hemolysis, raise the levels of analytes and cause

interferences. The position of the patient (standing, lying, seated) can affect the hematocrit and hence change the concentration of analytes. The tourniquet should be applied 3-4 inches above the site of venipuncture and should be released as soon as blood begins flowing into the collection device. The duration of venous occlusion (> 1 min) can affect the sample composition. Prolonged occlusion may result in Last update: 18-Nov-09 ©PXBioVisioN 2009 Page 2 / 5 hemoconcentration and subsequently resulting in an increase in miscellaneous analytes e.g. total protein levels. Blood should be collected from fasting patients in the morning between 7-9 am, because ingestion or circadian rhythms can alter the concentration of analytes considerably (e.g. total protein, hemoglobin, myoglobin).

1.1 Processing of blood samples

A quick separation of cells from the plasma is favourable, since cellular constituents may liberate substances that alter the composition of the sample. Generally, it is recommended that plasma and serum is centrifuged with 1300 to 2000 x g for 10 minutes within 30 min, after the collection of the sample. The temperature should generally be 15-24 °C, unless recommended differently for distinct analytes like gastrin or A-type natriuretic peptide. Processing at 4 °C appears to be attractive, becaus e enzymatic degradation processes are reduced at low temperatures. However, platelets become activated at low temperatures and release intracellular proteins and enzymes, which affect the sample composition. Thus, processing at low temperatures should be performed only after thrombocytes have been removed. Since one centrifugation step may not be sufficient enough for depletion of platelets below 10 cells/nL a second centrifugation step (2500 x g for 15 min at room temperature) or filtration step may be required to obtain platelet poor plasma. This procedure is only applicable to plasma since platelets in serum are already activated.

2 Materials

20 gauge needles and the appropriate adapter (e.g. Sarstedt, Nümbrecht, Germany) or a Vacutainer system (BD bioscience, Franklin Lakes, USA)

· alcohol (2-propanol) in spray flask

· swabs

· examination gloves

· blood collection tubes (e.g. Sarstedt, Nümbrecht, Germany)

· centrifuge with a swinging bucket rotor (e.g. Sigma 4K15, Sigma Laborzentrifugen, Osterode, Harz)

· 10 mL syringe equipped with a cellulose acetate filter unit with 0.2 µm pore size and 5 cm2 filtration area (e.g. Sartorius Minisart, Sarstedt)

· 2 mL cryo-vials

· pipette and tips

3 METHODS

Venipuncture of a orbital vein is performed using a 20 gauge needle (diameter: 0.9mm, e.g. butterfly system max. tubing length: 6 cm). If a tourniquet is applied, it should not remain in place for longer than 1 minute (risk of falsifying results due to hemoconcentration). As soon as the blood flows into the container, the tourniquet has to be released at least partially. If more time is required, the tourniquet has to be released so that circulation resumes and normal skin colour returns to the extremity. Prior to blood collection for proteomics analysis, blood is aspirated into a first container (e.g. 2.7 mL S-Monovette, Sarstedt, Nümbrecht, Germany). This is done to flush all surfaces and remove initial traces of contact induced coagulation. This sample is not useful for analysis. Afterwards, blood is drawn into a standard EDTA or citrate containing syringe (e. g. 9 mL EDTA-Monovette, Sarstedt, Nümbrecht, Germany). Depending on ease of blood flow, several samples can be collected. Free flow with mild aspiration has to be assured to avoid haemolysis. After venipuncture, plasma is obtained by centrifugation for 10 min at 2000 x g at room temperature. This centrifugation has to be started in within 30 min after blood collection. The resulting plasma sample has now been separated from red and white blood cells in an efficient and gentle way. Nevertheless, a significant number of platelets (~25%) is still present in the sample. This requires an additional preparation step. For a second centrifugation step to remove platelets the plasma sample is transferred into a second vial and centrifuged for 15 min at 2500 x g at room temperature. After this centrifugation, the supernatant is transferred in aliquots of 1.5 mL into cryo vials. Samples are transferred to a –80°C freezer in withi n 30 min. Storage is at –80°C, transport of samples is done on dry ice.

4 Notes

4.1 Frequently made mistakes

4.1.1 Blood withdrawal

· The Patient was not fasting (i.e. had eaten prior to sampling).

· The blood was drawn from an infusive line.

· The blood was drawn in other position (e.g. supine, upright).

· The consumables used were different to those recommended.

· The expiry date of consumables was already reached.

· The tubes were not properly filled.

· The tubes were agitated vigorously (shaken instead of gentle movement to dissolve the anticoagulant).

· The blood sample tubes were not consistently kept at room temperature.

· The sample tubes were put on ice or in a refrigerator.

4.1.2 Lab handling

· The centrifugation was delayed more than 30 min after the blood withdrawal.

· A cooling centrifuge was adjusted below room temperature.

· The centrifugation speed was wrong (e.g. rounds per minute were set instead of gforce).

· The centrifugation time was wrong.

· The removal of blood plasma by pipetting was done without proper caution.
Consequently the Buffy coat or the red blood cells were churned up.

· The second centrifugation of recovered plasma samples was delayed after end of first centrifugation.

4.1.3 Storage of samples

· The storage of samples was delayed.

· The storage temperatures were above -80°C.

· The labelling of sample containers is unreadable or confusable.

· The attachment of the labels to the sample containers was not sufficient during storage or handling resulting in lost of labels. Last update:18-Nov-09 ©PXBioVisioN 2009 Page 5 / 5

4.2 General recommendations

· A proper first centrifugation should produce a visible white blood cell layer (buffy coat) between red blood cells and plasma. If not, centrifugation speed or time may be wrong

· One should discard plasma that is icteric or exhibit signs of haemolysis. One should check with specialist if this may due to that particular disease.

Bile duct cannulation

Bile juice

Bile is essential to mammalian health. It is composed of water, bile acids (taurocholic acid, glycocholic acid), bilirubin, phospholipids, cholesterol, pancreatic juice (trypsinogen, other

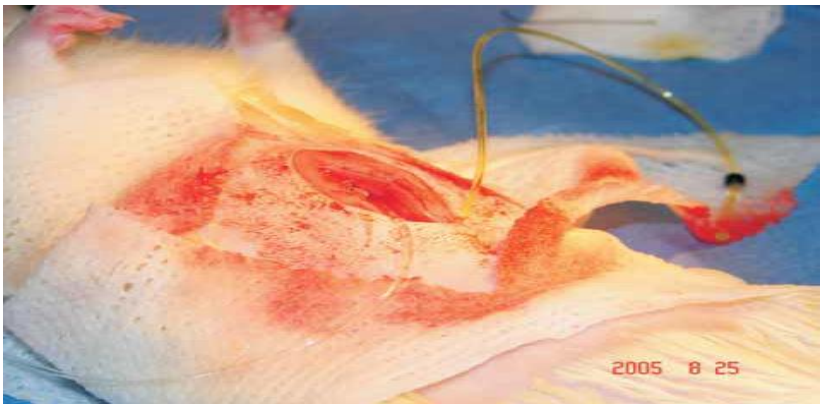
enzymes) and inorganic salts. Bile acids are responsible for the absorption of fat and fat-soluble vitamine from food in the gastrointestinal tract. Bile is vital for maintenance of cholesterol homeostasis. It is also bacteriocidal to microbes entering the body via food. The primary route for elimation of bilirubin is bile which transports it to the intestines and feces. One consequence of hepatic disease, or damage to hepatocytes by toxins or xenobiotics, is the failure of the liver to produce sufficient bile to efficiently eliminate bilirubin, resulting in jaundice. The potential to monitor bilirubin content in bile is therefore a potential safty pharmacology tool, although most work in this area has been done with isolated rat livers rather than repeat doses in living animals. An in vivo technique to monitor bilirubin content before and after multiple doses in the same subject would provide hepatotoxic screening. Bile flow responds to the enteric hormones **cholecystokinin and secretin** which also influence pancreatic function. Bile emulsifies lipids passing through the GI tract, activates lipases, and facilitates enterohepatic transport of drugs, metabolites and wastes. Bile salts are reabsorbed in the ileum and returned directly to the liver via the portal vein. Hepatocytes are so efficient at reabsorbing bile, that **95% of bile acids that were delivered to the duodenum are recycled**. Rats and horses are the only mammals lacking a gall bladder, which absorbs water and electrolytes from bile and conserves it during fasting periods.

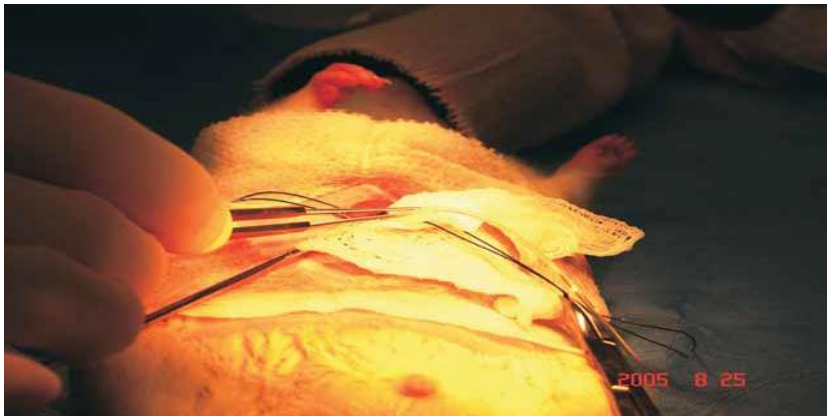
MATERIALS AND METHODS

Materials

The bile duct cannulae, length approximately 100-150mm (i.d. 0'5mm, o.d. 0'8mm) were prepared from medical grade clear vinyl tubing (Dural Plastics, Australia). A sleeve of silastic silicone tubing (Dow Corning, USA), length approximately 3mm (i.d. 0,64 mm, o.d. 1'19 mm) was fitted over one tapered end. The protective housing was prepared from perspex material (see Figs 1 and 2 for details). A perspex cap was screwed into the housing, when the animal was not under experimental use. When the rat was under experimental conditions, extension tubing connected to the cannulae, were protected by use of a modified sampling spring (Harvard Apparatus Ltd, UK) (see Fig. 2). A perspex cover was placed over the sampling spring which was then screwed into the perspex housing.

Surgical procedure





This surgical procedure was adapted from a published technique by Chipman and Cropper (1977). Barrier-reared, male SK&F Wistar rats (outbred, weight range 250-270 g) were housed on Grade 6 sawdust (Sawdust Marketing Company, Manea, Cambridgeshire) in polypropylene RCI cages (North Kent Plastics Ltd, Dartford, Kent; width O'31 m, height 0'18 m and length 0'495 m) and had access to food and water prior to surgery. Anaesthesia was induced using *4070* (v/v) halothane (lCI Ltd, Macclesfield, Cheshire) in oxygen and was maintained at 1·5% (v/v) halothane in oxygen. The animal was prepared for surgery by clipping the hair in two regions, on the ventral and upper left side of the abdomen. These areas were then cleaned using *5OJo* (v/v) Roccal (Winthrop Labs, Guildford, Surrey) swabs. Cotton gauze drapes, moistened with warm sterile isotonic saline, were placed on the abdomen and a midline incision was made from the xiphisternum to the umbilicus. The duodenal loop was identified and exteriorized through the incision area, exposing the bile duct (see Fig. I for details). At all times during exposure, the duodenum and bile duct were kept moistened with warm sterile isotonic saline. A cannulation site was selected in an area free of major blood vessels, normally about one-third of the way along the bile duct from the duodenum. In this area, a loop of cotton thread was taken beneath the duct with forceps and cut to form the final ligatures. These were loosely tied, one being taken proximally and the

other distally. The bile duct was then incised using 'iris' scissors. Insertion of the cannula into the duct towards the liver was confirmed by observing the bile flow into the cannula. The proximal ligature was tightened just proximal to the sleeve, since overtightening could block the lumen. The flow was then reconfirmed. This procedure was repeated for insertion of the second cannula into the bile duct towards the duodenum. Insertion of this cannula was confirmed by injecting warm isotonic saline (approximately 0·2 ml) into the cannula and observing enlargement of the duodenal loop. The distal ligature was then tightened, as previously described. To secure the cannulae, each ligature was taken around the opposing cannula and tied again so that each cannula was anchored both above and below its sleeve, as shown in Fig. 1. The ligatures were trimmed and the duodenum was returned to the abdominal cavity. Bile flow was confirmed at each stage. simple sutures (4/0 mersilk). The skin was finally closed around the housing using simple interrupted sutures (4/0 mersilk). The laparotomy wound was closed in two layers, with a simple continuous suture (4/0 mersilk) to close the peritoneum and muscle, and a row of simple interrupted sutures to close the skin. The animal was allowed to recover for 4-5 days, during which time the drinking water was supplemented *5070* (w/v) glucose, 0,9% (w/v) NaCI and O' 5% (w/v) KCI. Free access to food was allowed at all times. Animals were weighed and examined daily for health and bile flow during the recovery period, which was normally 7 days. If the animals showed signs of ill-health, pain or distress, they were humanely killed by an overdose of anaesthetic.

Animal housing: Animal rooms were maintained at 21 ± 2 cC, 40-60% r.h., with a 12h light period commencing at 0700 h GMT. After surgery, animals were housed singly in plastic polypropylene RCI cages on Grade 6 sawdust (Sawdust Marketing Company, Manea, Cambridgeshire). They were allowed food [PRD pelletted diet (Biosure Ltd, Manea, Cambridgeshire)] and water *ad libitum* and had their bedding changed on a regular basis. Under experimental conditions these animals were housed in glass metabolism cages (Jencons Ltd, Hemel Hempstead, Hertfordshire) with free access to food and water. The animal was then turned on its side and an incision was made in the left side. The abdominal wall was then incised from the peritoneal side in an area free of major blood vessels. The cannulae was exteriorized through the incision and connected together to form a loop, using silastic tubing. This loop was protected in a perspex housing (see Fig. I) which was attached to the animal by securing the base plate within the abdominal cavity. The abdominal wall was closed using a simple suture (4/0 mersilk). The housing was then anchored to the abdominal wall using four.

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