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RESEARCH PAPER

One Mouse, One Pharmacokinetic Profile: Quantitative Whole Blood Serial Sampling for Biotherapeutics

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ABSTRACT

Purpose The purpose of this study was to validate the approach of serial sampling from one mouse through ligand binding assay (LBA) quantification of dosed biotherapeutic in diluted whole blood to derive a pharmacokinetic (PK) profile.

Methods This investigation compared PK parameters obtained using serial and composite sampling methods following administration of human IgG monoclonal antibody. The serial sampling technique was established by collecting 10 μL of blood via tail vein at each time point following drug administration. Blood was immediately diluted into buffer followed by analyte quantitation using Gyrolab to derive plasma concentrations. Additional studies were conducted to understand matrix and sampling site effects on drug concentrations.

Results The drug concentration profiles, irrespective of biological matrix, and PK parameters using both sampling methods were not significantly different. There were no sampling site effects on drug concentration measurements except that concentrations were slightly lower in sodium citrated plasma than other matrices.

Conclusions We recommend the application of mouse serial sampling, particularly with limiting drug supply or specialized animal models. Overall the efficiencies gained by serial sampling were 40–80% savings in study cost, animal usage, study length and drug conservation while inter-subject variability across PK parameters was less than 30%.

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
DBS	Dried blood spot
HCT	Hematocrit
IP	Intra-peritoneal
IV	Intravenous
LBA	Ligand binding assay
PK	Pharmacokinetics
PMT	Photomultiplier tube
RO	Retro-orbital

INTRODUCTION

Pharmacokinetics (PK) data provide a time course of drug concentrations in circulation, and are important in evaluation of new biotherapeutics in drug development. In the early phases of drug discovery, preclinical PK studies are typically carried out in mice. Their small body size imposes physiological restrictions on blood sampling volume, leading to insufficient amounts of sample available for multiple analytical measurements such as drug, biomarker, or clinical chemistry profiles. As such, traditional composite sampling is often used, where time points across a time course are taken from different animals, providing sufficient volume for bioanalytical analyses. Composite sampling is often taken from different sites of the animal, which could lead to variances in measured analyte concentrations. Moreover, this approach requires greater numbers of animals and as a direct consequence, greater amounts of drug material with associated resource costs (animal husbandry and human resources) to support a study. In addition, inter-animal variability cannot be well controlled using composite sampling technique.

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Serial sampling is an alternative approach in which all PK time points are collected from a single dosed animal and from one collection site. Serial sampling has been extensively used for Dried Blood Spot (DBS) analysis, where a small amount of blood is collected from a mouse and dried as a "spot" on special sample cards (1–4). From an ethical perspective, this approach is supported by the 3 Rs of animal welfare (reduce, refine and replace) (5). The 3 Rs approach seeks to reduce animal resources used for scientific experimentation, reduce the pain and suffering of animals that are utilized, and replace *in vivo* studies with *in vitro* systems wherever possible.

The serial sampling technique reduces animal resources since a full PK profile is obtained from each individually dosed mouse. There are also financial benefits to this approach on several fronts. Animal resources, cost of animal housing, husbandry and biohazard/sharps waste production are significantly reduced. The amount of dosing compound required is decreased, adding to the cost savings. In addition, when specialized or rare animals are needed for a PK study, these may be expensive or difficult to acquire, and the benefit of using serial sampling is even more dramatic.

Finally, the scientific advantages to this approach are substantial. Since a full PK profile is obtained from each individual animal using serial sampling, the inter-subject variability in these data are more controlled than with composite sampling, in which a single PK profile is obtained by combining concentrations from various animals. Less variability in preclinical PK studies translates to better quality data, and subsequently more accurate dose predictions in humans.

For the above stated reasons, serial sampling for PK studies is a beneficial approach for bioanalytical laboratories. However, the small sample volume obtained (~ 10 µL of blood) cannot be accommodated by most LBA platforms for drug quantitation. The recent availability of the Gyrolab instrument allows small sample volume capability (<10 µL), conserving both sample volume and assay critical reagents, while retaining similar assay sensitivity (low ng/mL range) to most other LBA platforms. The small sample volume, large assay dynamic range, automation capabilities and tolerance to different matrices also makes Gyrolab an attractive platform to support PK studies using this sampling method (6,7).

Blood collected via serial sampling can be diluted or processed to different matrices including serum or plasma. However, only ~ 5 µL of serum or plasma will be acquired from ~ 10 µL of blood sample which is not ideal for storing multiple aliquots for repeat analysis or additional bioanalytical measurements. Whole blood is a common matrix used in DBS analysis of biotherapeutics, and has also been used for LC/MS quantitation of both large and small molecules (1), but has not been routinely used in LBAs. Quantitating drug concentrations in diluted whole blood could have several advantages over serum or plasma matrix in serial sampling. Diluting the blood in assay buffer after collection provides a larger volume of sample to make multiple aliquots for reassay, or testing in other assay formats (e.g., biomarker measurements). Diluting the analyte in buffer immediately following sample collection could also dilute out potential proteolytic enzymes that could affect biotherapeutic stability. Finally, using diluted whole blood as a matrix is more efficient, as it removes the clotting or anticoagulant steps found in serum and plasma collection, avoiding anticoagulant additives that can affect drug detection in a sample. When comparing drug concentrations in whole blood *versus* serum or plasma, a hematocrit (HCT) correction factor must be applied to account for the volume in the aqueous solution taken up by blood cells. A single corrective factor can be used since it has been demonstrated that hematocrit factor does not vary much between mice, irrespective of the sample collection site (8).

The stress on animals imposed through the blood collection process and/or use of anesthesia can also change biochemical processes *in vivo*, including upregulation of acute stress indicators such as adrenocorticotropic hormone (ACTH) and corticosterone. It has been reported that levels of these stress hormones is significantly reduced in tail sampling when compared with vena facialis and saphenous vein sampling (9). In addition, the site of sample collection may also affect the drug concentration measured in a sample (9–11). In composite sampling, different sites of collection may be used, such as retro-orbital (RO) bleeds and cardiac puncture, which can add to data variability, whereas serial sampling uses just one site of collection, typically the tail or saphenous vein.

The goal of this work was to compare a PK time concentration profile of a human IgG control antibody using serial sampling via tail bleeding to the traditional composite sampling from different sites of collection; RO and cardiac. In addition, diluted whole blood matrix (serial bleeding) *vs.* plasma (composite sampling), using different anticoagulants (EDTA and sodium citrate) and serum were evaluated to assess sample processing and the use of different matrices to quantitate the drug. Finally, the site of sample collection was also examined, comparing RO, tail and cardiac sampling sites from the same individual animals. Two applicable case studies are highlighted applying the serial sampling technique. Case Study 1 involved the rank ordering of potential drug candidates using PK profiles in discovery, where minimal drug material was available for dosing. Case Study 2 entailed the use of an expensive genetically engineered mouse model, where conservation of animal resources and cost was essential. Inter-subject variability and PK parameters using serial sampling for both case studies were also evaluated.

MATERIALS AND METHODS

Table I summarizes all the experiments described in this manuscript.

Table I Summary of *In Vivo* and *In Vitro* Experiments

Experiment	Description	Data
PK study (*in vivo*)	Compare tail serial *vs* composite RO and cardiac sampling	Table II, Figs. 1 and 2
Hematocrit	Compare hematocrit in tail and cardiac bleeds (data described in text)	In Results section
In vitro recovery study and hematocrit	Spike recovery of drug in whole blood; processed to serum, EDTA plasma and diluted whole blood (hematocrit tested for calculation of % recovery)	Table III
Effect of sampling site and anti-coagulant (*in vivo*)	Compare drug concentrations from tail, RO and cardiac sites; compare sodium citrated *vs* EDTA plasma	Table IV, Fig. 3
Case Study 1 (*in vivo*)	Rank order of potential drug candidates	Table V, Fig. 4
Case Study 2 (*in vivo*)	Engineered mouse model PK study	Table VI, Fig. 5

Animal Studies

Pfizer Institutional Animal Care and Use Committee approved all aspects of these studies. All studies were performed in accordance with the National Institutes of Health guide for the care and use of animal resources. Male C57BL/6 mice were used in the sampling method feasibility PK study; male Balb/c mice were used in Case 1 PK study. Both C57BL/6 mice and Balb/c mice were purchased from Charles River Labs (Wilmington, MA). Male transgenic mice with C57BL/6 background purchased from Jackson Lab (Bar Harbor, ME) were used in Case 2 PK study. All the animals were 9–11 week old with body weight about 25 g. For all three PK studies, six mice per group received a single dose of test articles. In the sampling method feasibility PK studies, 10 mg/kg of human IgG control antibody was given via intra-peritoneal (IP) route. The blood was collected via serial tail sampling procedure and processed to diluted whole blood or collected via composite sampling procedure and processed to sodium citrated or EDTA plasma as in procedures described below. In Case 1 and Case 2 PK studies, six monoclonal antibodies (Ab1-6) at 5 mg/kg or Antibody A at 2 mg/kg were administered intravenously, respectively. The administered doses were based on the most recent scheduled body weights. The test articles were prepared in PBS and the dosing volume was 4 mL/kg. Blood samples were collected at 0, 30 min, 3, 6, 24 h, 2, 3, 4, 7, 14, 21 and 28 days post dose. Blood collection and processing are described in the next section. The decision to use serial sampling for Case 1 PK (Ab1-6) study was due to the fact that this was in the early stages of drug screening, and as such only a few milligrams of material was available. The serial sampling study only required less than 2 mg of each protein. The serial sampling technique was chosen for Case 2 PK (Antibody A) analysis because the mice for the animal studies were expensive genetically engineered animals and only 6 animals were required using serial sampling method as opposed to 15–30 animals using the traditional composite sampling method per study.

Blood Collection Procedure

Serial Tail Sampling Procedure

The diagram in Fig. 1 depicts stages of serial tail sampling procedure including pre-warming, serial tail sampling and sample preparation. After the mice were dosed, serial sampling was conducted to obtain 10 μL of blood from the tail vein at various time points. Briefly, animals were warmed with a heat lamp prior to sample collection. Animals were warmed by the heat lamp for 2 min with the heat lamp 16 in. from cage floor at temperature of 30°C. This process was done to increase vasodilatation in the tail and was not required but would decrease sample collection time and the possibility of clotting. Afterwards, animals were placed on cage top and manually restrained at base of tail. A 25 gauge needle was used to stick the tail and the blood was quickly collected using a 10 μL capillary tube and bulb dispenser (Drummond Scientific Company Microcap ®). Each time point required a tail stick. The first time point was collected at the distal end of the tail (towards the tip) and each subsequent time point was collected a few millimeters up from the previous stick towards the base of the tail. The total volume of blood drawn did not exceed 20% of the total blood volume. After collection, the blood was quickly dispensed into 90 μL of Rexxip A buffer (Gyros, Upsala, Sweden) followed by pipetting up and down 3–4 times to make sure all visible blood had been dispensed from the capillary tube. The diluted blood was gently vortexed and spun at 1500 g for 10 min. The supernatant was transferred to a new tube and frozen at −80°C until further analysis.

Composite Sampling (Retro-Orbital and Cardiac Puncture)

Cardiac Puncture. After the animal was euthanized with CO_2, up to 700 μL of blood sample was taken from the cardiac ventricle through the diaphragm. Blood was withdrawn slowly to prevent the heart collapsing.

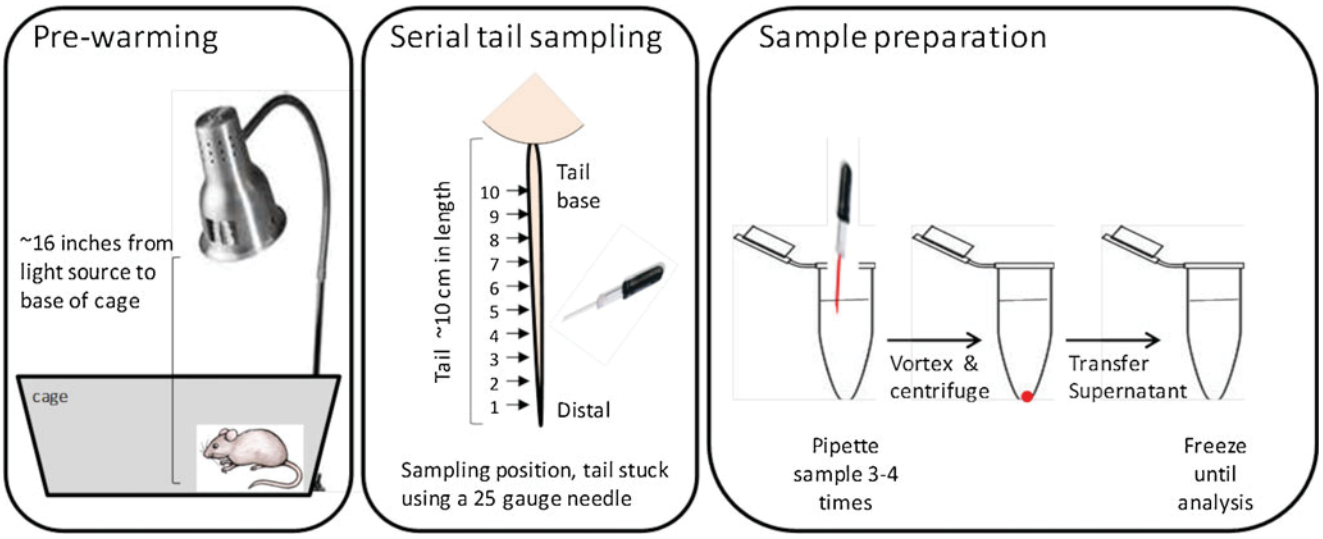

Fig. 1 Cartoon of serial sampling process. Cartoon depicts stages as pre-warming, serial tail sampling and sample preparation. Objects are not drawn to scale.

Retro-Orbital. After the animals were anesthetized, blood was collected from the venous sinus. The mouse was restrained, the neck gently scruffed and eye made to bulge. A capillary tube/pipette was inserted medially, laterally or dorsally. Blood was allowed to flow by capillary action into the capillary tube/pipette. The RO collection was conducted only once on each eye.

For plasma collection, the blood sample was collected into EDTA or sodium citrate tubes, and the tubes centrifuged at 1500 g for 10 min at room temperature. If a serum sample was to be harvested, no anticoagulant was utilized, and the blood sample was allowed to clot for 30 min followed by centrifugation at 1500 g for 10 min at room temperature. After centrifugation, plasma or serum sample was transferred to a new tube and stored at −80°C for further analysis.

Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated using non-compartmental methods with sparse sampling in Watson (Version 5.2, Thermo Fisher Scientific, Waltham, MA) where standard errors were also reported. Data in the terminal log-linear phase were analyzed by linear regression to estimate the terminal half-life ($t_{1/2}$). At least the last three time points were used to calculate $t_{1/2}$. Total AUC_{inf} was determined as the sum of AUC_{last} and AUC_{extrap}, where AUC_{last} was calculated from 0 to the last time point (t_{last}) with the last measurable concentration (C_{last}) using the linear trapezoidal rule and AUC_{extrap} was the extrapolated portion of the area from t_{last} to infinite using C_{last}/k. Total body clearance (CL) based on plasma concentrations was calculated as dose/AUC_{inf}, and the volume of distribution at steady-state (V_{dss}) was calculated as CL*AUMC/AUC_{inf}, where AUMC was the area under the first moment curve.

Hematocrit Test

After blood samples were collected via cardio puncture and tail bleed from the same animals ($N=3$), the capillary tube was filled with blood ½ to ¾ full using the end opposite to the colored ring. The clay sealer was used to seal the end opposite to the one used to fill it. Tubes were positioned opposite each other in the Readacrit Centrifuge (Becton, Dickinson and Company, Parsippany, NJ). Samples were centrifuged for 4 min, then placed on the reader scale to determine the % hematocrit.

In Vitro Recovery Study for Whole Blood, Serum and Plasma

Immediately after whole blood was collected from mice ($N=6$) via cardiac puncture, human IgG control antibody was spiked into the whole blood to a final concentration of 100 µg/mL. Samples were processed five different ways using the spiked-in whole blood:

1. Diluted whole blood sample: 10 µL capillary tube was filled with the spiked whole blood and the sample was transferred to an Eppendorf tube and mixed with 90 µL of Rexxip A Buffer. No centrifugation step was utilized for this processed sample.
2. Centrifuged diluted whole blood: Blood was collected as in (1) above, and the diluted whole blood was centrifuged by spinning at 1500 g for 10 min, and supernatant collected.
3. Serum sample: 20 µL capillary tube was filled with the spiked whole blood and allowed to clot at room temperature for at least 30 min. The filled capillary tube was centrifuged in Readacrit Centrifuge and serum was collected.
4. EDTA plasma sample: 20 µL capillary tube coated with EDTA was filled with the spiked whole blood. The filled

capillary tube was spun in Readacrit Centrifuge and the plasma was collected.

5. Hematocrit checking was conducted for the blood samples from all 6 animals following the procedure described above.

All collected samples were stored immediately at −80°C until further sample analysis.

Effect of Sampling Site and Anticoagulants

To understand the effect of different sample collection sites and different anticoagulants on measured drug concentration, 10 mg/kg of human IgG control antibody was given via IP route to 12 C57BL/6 mice. Blood was collected at 6 h (N=6) or 24 h post dose (N=6), respectively. From each mouse at 6 or 24 h, 10 μL of blood was collected from the tail and processed following the serial sampling procedure as described above. 200 μL of blood was collected following RO bleed procedure and cardiac puncture as described above. 90 μL of RO or terminal bleeds was added to 10 μL of 32% sodium citrate and processed to plasma and 100 μL of RO or terminal bleeds was added to EDTA coated tubes and processed to plasma for further LBA analysis.

LBA Method Using Gyrolab

Sample Analysis (Sampling Method Feasibility PK Studies)

PK and *in vitro* spiked samples were analyzed for human IgG control antibody concentrations using a Gyrolab platform LBA. The assay was qualified for use with serum, plasma and diluted whole blood matrix prior to running study samples. The human IgG control MAb reference standards and quality controls were prepared in Assay Buffer (0.2 M Tricine, 1% bovine serum albumin (BSA), 0.15 M NaCl, 0.01% Tween-20, 0.05% Proclin300, pH 9.5) and the study samples were diluted into the assay range of quantitation using Assay Buffer (minimum required dilution for all matrices tested was 1:10). Briefly, biotinylated goat anti-human IgG (H + L) (Bethyl Laboratories, Inc., Montgomery, Tx) was captured onto streptavidin coated beads located in the affinity capture column of the Gyrolab CD microstructure. After being captured onto the affinity capture column, bound human IgG control antibody in samples was detected with Alexa 647 labeled goat anti-human IgG (H + L) (Molecular Probes, Inc., Eugene, OR). The fluorescent signal on the column allowed for detection of the bound IgG. Response Units were read by the Gyrolab instrument at the 5% photomultiplier tube (PMT) setting. Sample concentrations were determined by interpolation from a standard curve that was fit using a 5-parameter logistic curve fit with $1/y^2$ response weighting using Gyrolab Evaluator Software (Version 3.1.5.137). The assay range of quantitation was 23.4 ng/mL – 3000 ng/mL in 100% C57BL/6 mouse plasma. Note that the concentrations were reported as plasma concentrations, even when diluted whole blood was used, in which case the hematocrit factor had to be accounted for and is described in the next section.

Sample Analysis (Case 1 PK Study)

Sample analysis was the same as that used for the sampling method feasibility PK studies, except that Rexxip A buffer (Gyros, Upsala, Sweden) was used as the Assay Buffer, and Response Units were read using a 1% PMT setting. The assay

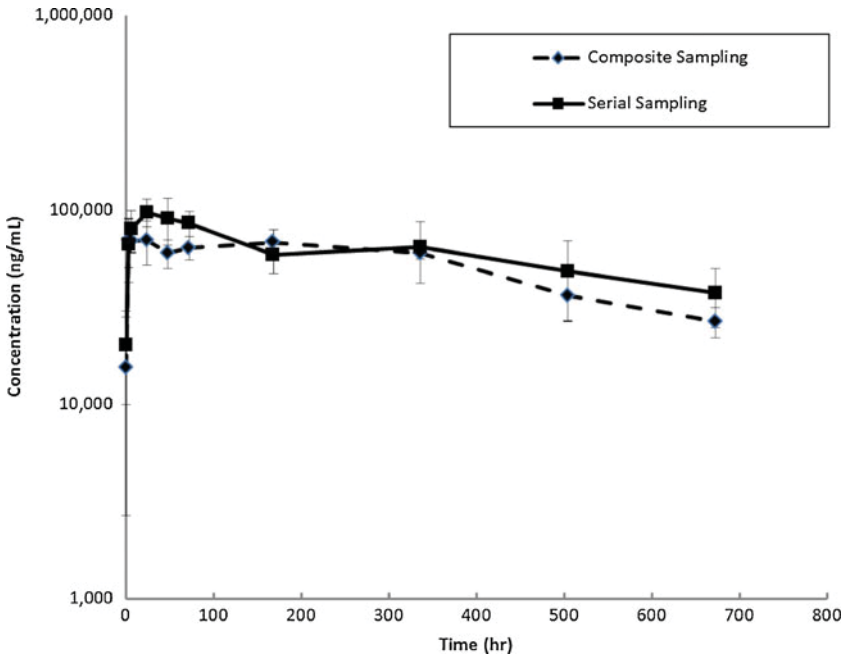

Fig. 2 Mouse PK profile (mean ± stdev, N=6) via serial sampling and composite sampling procedures following administration of 10 mg/kg of control IgG antibody via IP route. The diluted whole blood concentrations following serial sampling and sodium citrate plasma concentrations following composite sampling via RO bleeds and cardiac puncture were measured by Gyrolab LBA.

Table II Mouse IP PK Parameters via Serial Sampling and Composite Sampling Procedures ($N = 6$ for Each Group)

	Animal #	$t_{1/2}$ (h)	C_{max} (μg/mL)	T_{max} (h)	AUC_{last} (mg*h/mL)
Composite sampling	Composite	289	70	3	34.9
Serial sampling[a]	# 1	266	114	24	36
	# 2	460	100	24	43
	# 3	303	72	24	26
	# 4	297	123	48	52
	# 5	555	100	48	44
	Mean	376	102	34	40
	Stdev[b]	126	20	16	8
	CV%	29	19	47	21

[a] Animal #6 was excluded from PK analysis due to incomplete sample collection for this animal

[b] Stdev = standard deviation

range of quantitation was 41 ng/mL to 10,000 ng/mL in 100% Balb/c mouse plasma.

Sample Analysis (Case 2 PK Study)

Biotinylated mouse anti-human IgG (Fc) JDC-10 clone (Southern Biotech, Birmingham, AL) was used as the capture reagent. Alexa 647 labeled mouse anti-human IgG (Fc) JDC-10 was prepared in-house as per manufacturer's recommendations and used as the detector reagent. Rexxip A buffer (Gyros, Upsala, Sweden) was used as the assay buffer, and 1% PMT setting was utilized for Response Unit generation. The assay quantitative range was 91 – 55,552 ng/mL in 100% C57BL/6 mouse plasma.

Dilution Factor

The whole blood study samples obtained via tail serial sampling were collected at a 1:10 MRD in Rexxip A buffer (10 µL blood into 90 µL buffer). However, since plasma concentrations were the desired endpoint to compare across all matrices tested, a hematocrit component correction factor was applied for whole blood, using the following formula:

$$[\text{Drug}]_{\text{blood}}/(1-\text{hematocrit}) = [\text{Drug}]_{\text{plasma}}$$

The estimated average hematocrit in the mouse is 0.45 (12,13). Hence, in 10 µL of blood, 45% of the volume was attributed to cells and 55% was plasma volume. Therefore, theoretically only 5.5 µL plasma was collected into 90 µL buffer upon whole blood sample collection, and the dilution factor was actually 1:17.36, as opposed to 1:10.

Statistical Analysis

To determine if there was a significant difference in AUC_{inf} and $t_{1/2}$ between the composite sampling and serial sampling, statistical analysis was carried out with Microsoft Excel 2007 using a two sample Z-test. Statistical difference was defined as P value < 0.05.

RESULTS

PK Comparison Between Serial Sampling and Composite Sampling Studies

Pharmacokinetics of human IgG control antibody was determined in C57BL/6 mice following a single IP dose using both serial sampling and composite sampling procedures ($N = 6$). Animal #6 in the serial sampling group was excluded from PK analysis due to the incomplete sample collection time points for this animal. The concentration time profiles are illustrated in Fig. 2. The inter-subject variability (CV%) for concentrations at different time points from serial sampling

Table III *In Vitro* Drug Spike Recovery Comparison From Whole Blood, Serum and EDTA Plasma

Animal	HCT	% Recovery in Serum	% Recovery in EDTA plasma	% Recovery in centrifuged diluted whole blood[a]	% Recovery in centrifuged diluted whole blood[b]
Mouse 1	45%	99.4	98.2	89.1	89.1
Mouse 2	55%	98.6	96.8	99.1	120
Mouse 3	45%	104	104	102	102
Mouse 4	45%	113	103	105	105
Mouse 5	55%	108	95.1	105	127
Mouse 6	50%	106	106	102	111
Mean	49%	105	100	100	109
Stdev	5%	5	4	6	14
CV%	10	5	4	6	12

[a] %Recovery in centrifuged diluted whole blood based on the initial dilution factor of 17.36

[b] %Recovery in centrifuged diluted whole blood based on the actual HCT corrected dilution factor

Table IV Effect of Sampling Site and Anticoagulants on Analyte Concentrations

Collection site		Tail	RO[a]		Cardiac	
Time (h)	Conc. (μg/mL)	Diluted whole blood	Na Cit[b] plasma	EDTA plasma	Na Cit plasma	EDTA plasma
6	Mean	9.8	7.7	10.6	8.7	10.0
	Stdev	2.7	2.4	2.8	2.6	2.9
	CV%	27	37	26	30	29
24	Mean	10.8	8.6	10.0	8.5	9.3
	Stdev	0.8	0.8	1.0	0.2	0.9
	CV%	7	9	10	3	10

[a] *RO* retro-orbital

[b] *Na Cit* sodium citrate

ranged from 8 to 39% *vs.* 7–83% from composite sampling. After a 10 mg/kg IP dose, the plasma concentrations via composite sampling procedure and serial sampling procedure were very similar for most of the time points post dose except for 24, 48 and 72 h when the plasma concentrations via serial sampling procedure were higher than the sodium citrate plasma concentrations collected via composite sampling (P value < 0.05). The difference may be explained by the anticoagulant effect where sodium citrated plasma matrix results in lower human IgG concentrations than EDTA plasma as described in more detail in the following section. The terminal half-life $t_{1/2}$ was 289 and 376 ± 126 h; and AUC were 35 and 40 ± 8 mL/h/kg, from composite *versus* serial sampling methods, respectively (Table II). A two sample Z-test was conducted on $t_{1/2}$ and AUC, and P values were 0.06 and 0.08 from composite *versus* serial sampling methods, respectively. Thus, after either serial sampling or composite sampling procedures, the calculated PK parameters of human IgG control antibody was not significantly different.

Hematocrit Test

After the blood samples were collected via cardiac puncture and tail bleed from same animals ($N = 3$), a hematocrit test was conducted using both samples. The mean hematocrit was 46.0 ± 5.7 and 46.7 ± 1.4 for samples collected via tail bleed and cardio puncture, respectively. This was in agreement with the expected HCT of 45%.

Drug Recovery Comparison from Whole Blood, Serum and Plasma

Immediately after whole blood collection from mice ($N = 6$) via cardiac puncture, human IgG control antibody was spiked into the whole blood with a final concentration of 100 μg/mL. Four types of matrices were processed from the spiked-in

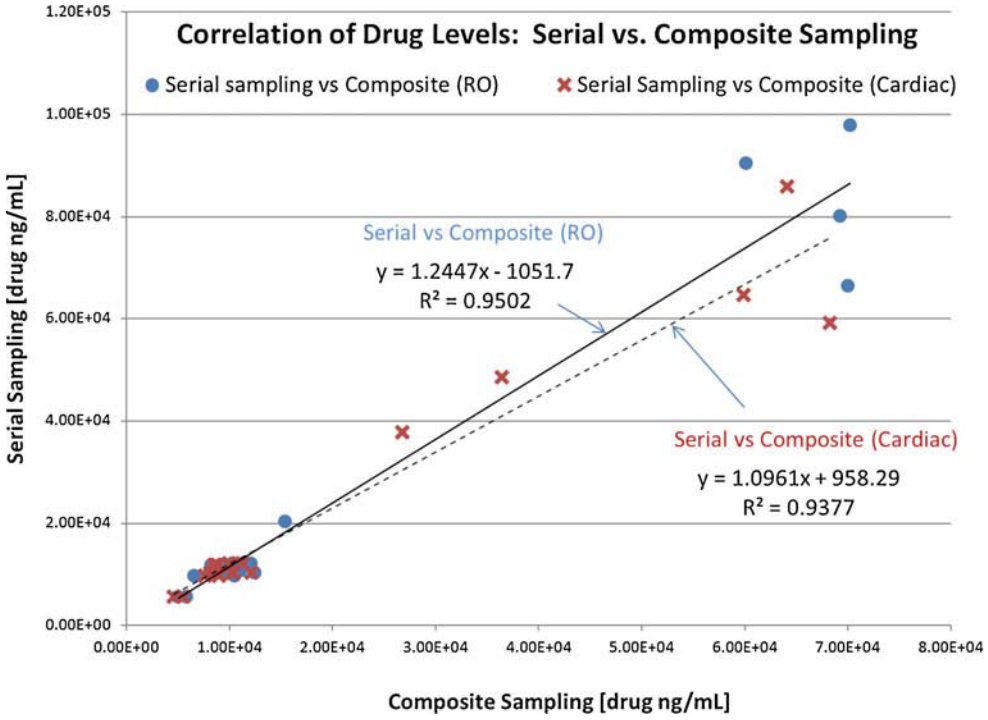

Fig. 3 Regression analysis of tail serial *vs.* composite sampling using RO and cardiac collection sites.

whole blood including; serum, EDTA plasma, centrifuged and uncentrifuged diluted whole blood (1:10 in Rexxip A buffer). The concentrations of the spiked-in samples were measured using Gryolab LBA and % recovery of spike concentration was calculated. The % recovery among the different types of matrices were similar: 105 ± 5%, 100 ± 6%, 100 ± 6%, 109 ± 14% for serum, EDTA plasma, centrifuged diluted whole blood with recovery calculated both by using a 17.36 dilution factor (estimated HCT) and a dilution factor using actual measured HCT from individual animals (Table III). The uncentrifuged diluted whole blood had excessive hemolysis and contained particulate matter thus analysis was not feasible. Overall, no difference was observed in drug recovery in different types of processed matrices.

Effect of Sampling Site and Anticoagulants

To understand the effect of different sample collection sites on drug concentration, and the effect of anticoagulants to plasma concentration, animals were dosed with 10 mg/kg of human

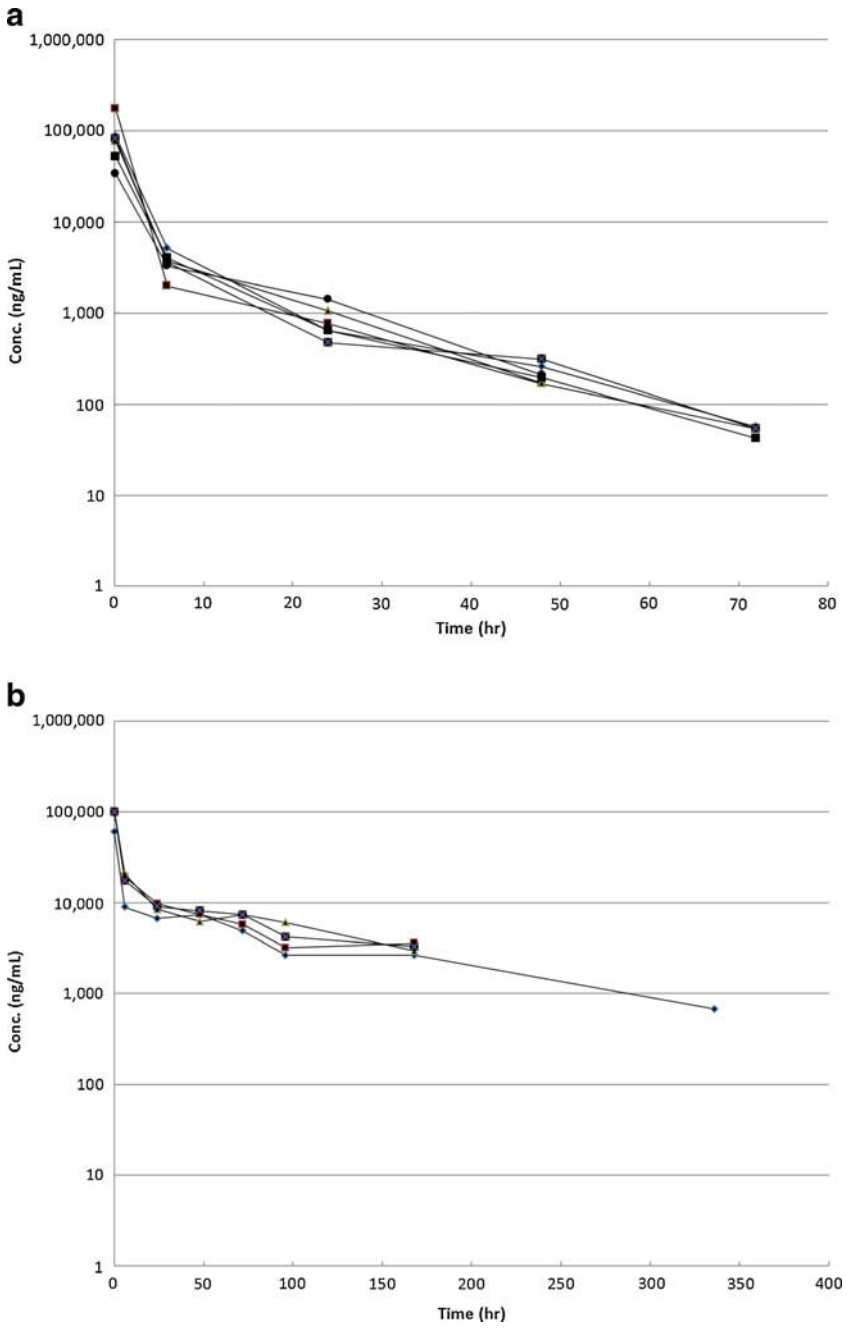

Fig. 4 Representative PK profiles of individual animals for Case 1 PK Study for Ab1 (**a**) and Ab5 (**b**). Five mg/kg of antibody 1–6 was administered to Balb/c mice via IV route and diluted whole blood was collected via serial sampling method and drug concentrations determined by Gyrolab LBA.

IgG control antibody via IP route, and samples collected at 6 h and 24 h. Blood was collected from tail, venous sinus (RO) and cardiac puncture (terminal) from the same animal and the collected blood was split into two parts and processed to sodium citrated plasma and EDTA plasma, respectively, and analyzed for drug concentration. At both 6 and 24 h, the measured drug concentrations from diluted blood from tail bleeds, EDTA plasma from RO and cardiac terminal bleeds were similar with average concentrations of approximately 10 µg/mL. However, sodium citrated plasma from both RO and cardiac terminal bleeds were lower with average concentrations of 7.7–8.7 µg/mL (Table IV), although the difference was not statistically different ($P=0.05$). Regression analysis (Fig. 3) was performed to compare drug concentrations obtained from tail serial sampling *vs* concentrations derived from RO and cardiac composite sampling, where correlation coefficient ($R^{2)}$ values were 0.9502 and 0.9377, respectively. The slope for the tail *vs* cardiac composite regression line was 1.096, demonstrating that the concentration values using the two sampling methods were equivalent. The slope of correlation between serial sampling and RO sampling was slightly higher at 1.245, which is consistent with the observed lower concentration values obtained with sodium citrate anticoagulant matrix used for composite sampling. The concentration differences found were also within the variability of the assay and therefore, sampling site and type of anticoagulant did not significantly impact drug concentration in the sample. Although sodium citrated plasma was not included in the in vitro drug spiking experiment, spike recovery was evaluated in this matrix during assay qualification. Six individual mouse sodium citrated plasma samples were spiked at the ULOQ and LLOQ levels for selectivity testing, and % recovery of spikes for the six individuals were within our acceptance criteria for non-regulated bioanalysis (at least 80% of the individuals must recover within +/−30% of nominal).

Case Studies

In Case 1 PK study, 5 mg/kg of each of the six monoclonal antibodies (Ab1-6) were dosed to 6 mice via IV route (on the same day) followed by tail vein serial sampling for concentration analysis by GyroLab LBA. In Case 2 PK study, 2 mg/kg Antibody A was dosed to 6 genetically engineered mice via IV route. Representative plasma time concentration PK profiles for individual animals from Case study 1 and Case study 2 are illustrated in Figs. 4 and 5. The inter-subject variability for Case study 1 and 2 were low in the serial sampling approach (CV% 9–50% and 15–40%, respectively) compared to the range from traditional composite sampling method based on historical PK data from our laboratory with CV% 5–150%. PK parameters were determined for all individual animals in each group. For Case 1 PK study (Table V), Ab6 had the lowest CL of 0.3 ml/h/kg and Ab1 had the highest CL of 18 ml/h/kg. Ab5 had relatively higher inter-subject variability (Fig. 4b) because the concentrations had a significant drop at approximately 336 h post dose possibly due to the formation of anti-drug antibodies. The CV% was less than 50% for PK parameters in Case 1 PK study. For Case 2 PK study, CV% range for all PK parameters evaluated was also low at less than 30% (Table VI).

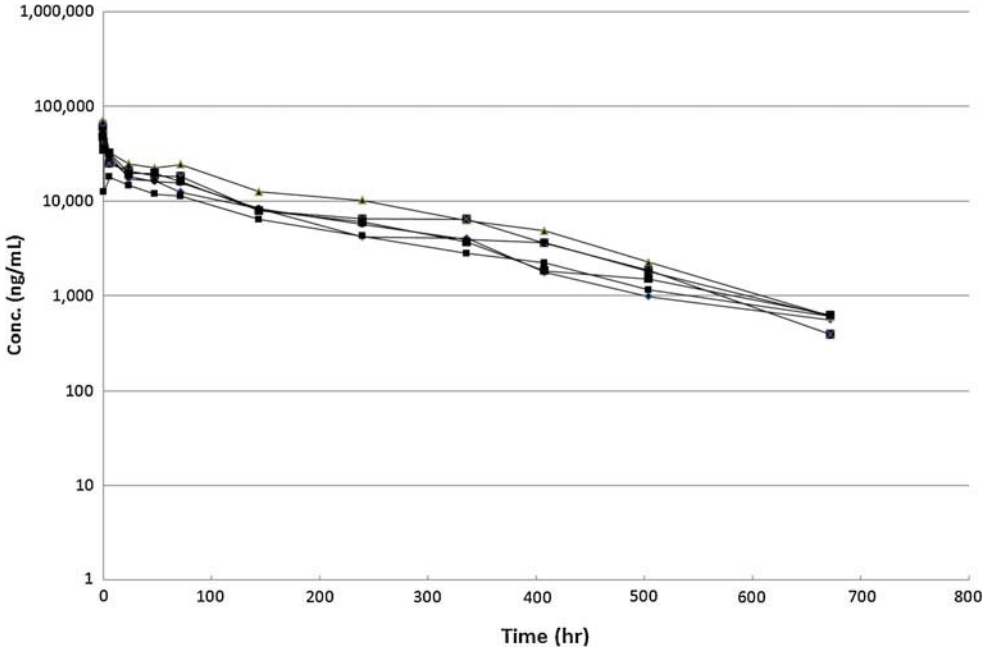

Fig. 5 PK profiles of individual animals for Case 2 PK study. Two mg/kg of antibody A was administered to a strain of transgenic mice via IV route and diluted whole blood was collected via serial sampling method and drug concentrations determined by Gyrolab LBA.

Table V PK Parameters for Case 1 PK Study in which 5 mg/kg Ab1-6 Were Dosed to Male Balb/c Mice Via IV Route and Serial Blood Sampling was Collected Via Tail ($N = 6$ Per Group)

Parameter[a]	Units	Ab1	Ab2	Ab3	Ab4	Ab5	Ab6
AUC_{inf}	μg*h/mL	323 ± 141	579 ± 82	548 ± 66	364 ± 55	1288 ± 147	5955 ± 1709
Co	μg/mL	92 ± 56	70 ± 10	58 ± 21	72 ± 28	95 ± 18	140 ± 81
$t_{1/2}$	h	173 ± 2	32 ± 3	42 ± 21	11 ± 1	97 ± 47	173 ± 53
CL	mL/h/kg	18 ± 7	9 ± 1	9 ± 1	14 ± 2	3 ± 1	0.3 ± 0.1

[a] Numbers are the average of six animals following intravenous administration of 5 mg/kg proteins

Cost of Savings

The implementation of serial sampling in two PK case studies was successful. For Case 1 PK study, the goal was to rank order six constructs based on PK profile, with limited material available for each biotherapeutic construct. Using the serial sampling method, only 36 mice were used instead of 180 mice required if using the traditional composite method. The six PK profiles were conducted simultaneously instead of sequentially if using the traditional method. Overall, there was 80% savings in number of animals used, 75% savings in drug material required, and 40% savings in the length of time for the in-life study. For Case 2 PK study, expensive and rare genetically engineered animals were required and utilization of serial sampling reduced the number of animals needed by 80% while concurrently generating high quality PK data, an improvement over what would be expected from composite sampling technique. It should be noted that the outright cost of doing bioanalysis using Gyrolab is greater than using a plate-based ligand binding assay platform such as ELISA given the instrument purchase and cost of CD's. However, Gyrolab brings efficiency with short assay time and automated back-end, saving analyst time which directly translates to cost savings (14). In addition, the broad dynamic range of the platform reduces the need for reassays compared to ELISA (also time savings), and reagent costs are reduced due to the small volume requirements.

DISCUSSION

With the availability of the Gyrolab platform that enables concentration analysis with as low as 10 μL of sample volume, the use of tail vein serial sampling technique to collect a complete PK time course using one mouse was explored and validated. This study investigated the difference in drug concentrations obtained from serial sampling with traditional composite method of sampling; the difference in drug concentration in different matrices (whole blood *vs.* serum *vs.* plasma) including potential differences across multiple anticoagulants (EDTA *vs.* sodium citrate); and the effect of sample processing and sampling site on drug concentration. It was demonstrated that drug concentrations of samples collected from cardiac puncture, RO or tail vein were similar; and the drug collected in whole blood, serum and EDTA plasma gave similar concentrations. From the sampling site and anticoagulant evaluation, it is interesting to note that sodium citrated plasma gave ~5–10% lower concentration (after correcting for sodium citrate dilution) than the other three matrices, though this was not statistically significant and was within expected assay variability. The anticoagulant effect may also explain the observed lower concentrations in most of the sodium citrated plasma samples from the PK study using composite sampling when compared with concentrations obtained from serial sampling. However, the PK parameters obtained from both methods did not show a statistical difference.

Whole blood sampling is a common matrix used in DBS analysis of biotherapeutics and biopharmaceuticals, and has also been used for LC/MS quantitation of both biologics and small molecules (1), but has not been routinely used in LBAs. For PK profiling, up to 10 time points is necessary and using serial sampling only 10 μL of blood from one mouse can be collected at each time point yielding up to 5 μL of serum or plasma. This small volume is not ideal for storing multiple aliquots for repeat analysis or additional bioanalytical measurements. This investigation has demonstrated that diluted whole blood (1:10, v:v) in assay buffer is an alternative matrix that provides higher volume for analysis, long-term storage of multiple aliquots and possible repeat measurements. In this feasibility study, plasma concentrations were calculated using a dilution factor of 17.36 from the measured concentration

Table VI PK Parameters for Case study 2 in Which 2 mg/kg Antibody A was Dosed to Transgenic Mice Via IV Route and Serial Blood Sampling was Collected Via Tail ($N = 6$ Per Group)

Parameter[a]	Units	Mean	Stdev	%CV
AUC_{inf}	μg*h/mL	173	43	25
Co	μg/mL	59	14	24
$t_{1/2}$	h	5	1	18
CL	mL/h/kg	12	3	23

[a] Numbers are the average of six animals following intravenous administration of 2 mg/kg proteins

that takes into account a hematocrit value of reported as 45% in mouse blood, similar to that measured by both RO bleeds and cardiac puncture.

From a bioanalytical perspective, the Gyrolab platform provided the small volume capability that adequately fulfills the needs of preclinical PK studies using serial sampling technique. As with any platform, assay requirements should be discussed so that appropriate methods are chosen before executing the study. In addition, it is recommended that the following are confirmed before study start: reagent availability, potential compound carry-over issues have been evaluated and mitigated during assay development, assay buffer composition and platform are compatible and the assay is suitable for its intended use with respect to quantitative range, sensitivity, precision and accuracy. If a Gyrolab is unavailable, this does not preclude the use of serial sampling, since other ligand-binding assay platforms, such as ELISA or Meso Scale Discovery®, may be utilized. Since volume requirements on these LBA platforms are greater than Gyrolab, the minimum required dilution may be increased to reduce the amount of blood needed, with the caveat that sensitivity would suffer as a result. Some other alternatives to Gyrolab for doing mouse serial sampling bioanalysis would be 1) the use of 384-well plates, 2) using a CRO with Gyrolab capabilities or 3) to use other emerging technologies with high sensitivity or small volume capabilities so less sample volume is required, e.g. immunoPCR, Optimiser™, Singulex Erenna® or Quanterix Simoa™ platforms. The serial sampling technique is an innovative and progressive alternative to traditional mouse PK and contributes to the design of humane animal research studies in accordance with the 3 Rs of animal welfare. This technique reduces the total number of animals needed by 60–80% to perform a study. Consequently, the size of maintained animal colonies, husbandry needs, facility space, and even the sharps and biohazard waste production was significantly reduced, reducing the overall cost of a PK study by more than 80%. The serial sampling via tail sticking is a very simple technique to learn and to teach. The overall inter-subject variability in sample concentrations and PK parameters are significantly reduced and the quality of the PK study data ultimately used in human dose prediction is greatly improved. The technique is especially useful when 1) test article is in short supply; 2) when rank ordering by PK of more than 3 test articles; and 3) when the animals utilized in the study are rare species, difficult models to develop or very expensive such as diseased animal models or genetically engineered and 4) when a small volume analysis LBA platform, such as Gyrolab, is available for drug quantitation. Thus, mouse serial sampling is a practical, efficient technique to obtain quality PK data for biotherapeutic drug development.

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