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Reduction of animal usage by serial bleeding of mice for pharmacokinetic studies: application of robotic sample preparation and fast liquid chromatography–mass spectrometry

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Abstract

Typically, pharmacokinetic studies in mice require one animal per time point, thus resulting in differences due to dosing error, animal to animal variation and more importantly the euthanasia of a large number of animals. A method for the determination of pharmacokinetic data from serially bled mice to support early drug discovery is described. Sample analysis relies on liquid chromatography coupled with tandem mass spectrometry permitting robust and reproducible analysis requiring approximately 3 min per sample. Several parameters are discussed including the method of sample collection, preparation and analysis. The use of serially bled mice has lead to a remarkable reduction in animal usage and a corresponding reduction in compound required for such experiments. Using conventional methodology, a nine-point pharmacokinetic curve with four animals per time point would require 36 mice. With the method described below, only four mice in total are used and euthanasia is not required, permitting reuse after several weeks recovery and washout. Also, pharmacodynamic–pharmacokinetic correlation is possible and is demonstrated using a mouse model of diabetes.

Keywords: Robotic sample preparation; Animal reduction; Pharmacokinetic screens; Serial bleeding

1. Introduction

The mouse is rapidly becoming the model of human genetics and physiology and is used extensively for animal models of human disease [1]. The ability to develop strains of mice that mimic human disease, as well as the prolific nature of these rodents makes them ideally suited to pharmacological study. Correlation of in vivo efficacy with blood concentration (pharmacodynamic–pharmacokinetic, PD–PK) of new chemical entities is an important aspect of drug development. Also, the ability to quickly generate pharmacokinetic data early in discovery is an essential requirement for the development of new drugs. While full pharmacokinetic data is valuable as compounds approach the refinement necessary for acceptance for usage in humans, such detail is often not required early in discovery. A quick snapshot of the disposition of the compound (C_{max}, ~$t_{1/2}$) is

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usually sufficient to rank new compounds relative to a lead compound. This information provides the medicinal chemists with direction for optimising lead compounds in order to develop a compound suitable for once a day dosing. Typically, a group of mice are euthanized for blood collection at each time point for pharmacokinetic studies and parallel groups of treated animals are used for in vivo pharmacological studies. Correlating efficacy with in vivo blood levels is based on the assumption that the study group has similar drug levels as the control group. There can be considerable animal-to-animal variation associated with this type of approach and euthanasia of a large number of animals has ethical and practical considerations. This is especially true when gene knockout or other valuable mice strains are being used for PK studies. Unfortunately, serial bleeding of mice has not been practical given the small volumes of blood, hence the small volume of plasma, available from these rodents.

For pharmacokinetic determinations, whole blood collected from the dosed animal is centrifuged to separate the plasma from the red blood cells. The plasma is then analyzed directly [2], after on-line [3] or off-line [4] solid-phase extraction, liquid–liquid extraction [5], or protein precipitation [6]. These experiments require a relatively large volume (>100 μl) of whole blood in order to produce enough plasma for analysis using liquid chromatography–mass spectrometry (LC–MS).

Recently, a method was reported for obtaining PK data from serially bled mice using capillary LC–MS [7]. In this approach, a small volume of whole blood (20 μl) was treated with acetonitrile (50 μl), centrifuged for 1 h, and the supernatant was evaporated to dryness. The resuspended sample (20 μl) was injected (1 μl) onto a capillary LC column allowing separation prior to MS analysis. The use of a capillary column resulted in limited lifetime, longer run times and a decrease in robustness relative to conventional methods of analysis. We have developed a method that requires only 10 μl of whole blood and minimal sample preparation. The sample preparation is automated using a commercially available workstation (Biomek 2000). The LC–MS analysis uses standard conditions typically employed when analyzing plasma samples processed in the usual manner (i.e., acetonitrile precipitation). This technique provides for direct correlation of in vivo efficacy in animal models of human disease with blood levels of the drug under study. As important is the substantial reduction in the number of animals required to perform routine PK studies on new compounds. This method implements two of the Russell–Burch ''3R'' tenet of ''Replacement, Reduction and Refinement'' for the use of animals in research [8].

2. Experimental

2.1. Reagents and materials

Compounds under study were synthesized at Merck Frosst Canada & Co. All solvents were HPLC grade; ammonium acetate (99.999%) and trisodium citrate were from Sigma (St. Louis, MO, USA). Luna columns were purchased from Phenomenex (Torrance, CA, USA).

2.2. Animals

Male C57BL/6J (B6) mice were obtained from Taconic Farm (Germantown, NY, USA) at 6–8 weeks of age. They were group-housed in a temperature and humidity controlled room and were put on a high-fat diet composed of 35% (w/w) fat supplied by BioServ (NJ, USA). An age-matched group of mice were given a normal diet composed of 5% (w/w) fat. After 6–9 weeks on the diet, the mice were used for experiments as described below.

2.3. Study design and oral glucose tolerance test

All procedures were approved by the Animal Care Committee at the Merck Frosst Center for Therapeutic Research according to guidelines established by the Canadian Council on Animal Care. A typical PK study involved the oral dosing of 4 C57BL/6J mice fed on a high-fat diet with the compound of interest. The compound was dosed to each animal at typically 100 mg/kg and blood was collected at 0, 0.25, 0.5, 1, 2, 4, 6, 8 and 10 h post-dosing. For the pharmacological efficacy studies fasted mice that had been on either a high-fat diet or normal diet were dosed orally at either 10 or 30 mg/kg 30 min prior to oral

administration of a glucose solution at 2 g/kg. Serial tail bled blood samples were collected 30 min prior to dosing and 1, 3 and 5 h post-dosing for measurement of drug levels. Tail blood samples (5 μl) were also collected at various time points (see Fig. 3A) for measurement of blood glucose using a glucometer (Precision Q-I-D; Abbott Labs.) and Medisense electrochemical test strips.

2.4. Sample collection and preparation

The protocol for sample preparation for pharmacokinetic studies is shown in Table 1. Most steps were automated using a Beckman Biomek 2000 workstation. Restrained animals were tail bled to produce a small drop of blood on an inverted weighing boat. A micropipette was used to accurately measure 10 μl of blood into a vial containing 30 μl of 0.1 *M* trisodium citrate. The sample and buffer were aspirated several times in order to rinse all the blood from the pipette tip. Serial bleeds of the same mouse were carried out at specified time intervals. Samples were collected into standard 2-ml screw-top high-performance liquid chromatography (HPLC) vials with separate 0.3 ml glass inserts. Vials were placed in Beckman 24 place racks with 13 mm (red) inserts for all sample-processing steps including centrifugation (Table 1). The precision and accuracy of the pipetting of the internal standard solution (10 μl) on the Biomek 2000 was ensured by using P20 pipettes for this operation. The addition of acetonitrile (50 μl) was carried out using P250 pipet tips. The relative standard deviation (RSD) of these transfers as determined by LC–MS of standard solutions was typically less than 10%. This is the total variation of the method including the injection and analysis by LC–MS. Typical errors in pipetting operations as defined by the manufacturer are less than 5% RSD. Given that animal to animal variation is expected to be much greater than this, it is an acceptable error.

Table 1
Sample preparation method

1. Pipet 30 μl of 0.1 *M* trisodium citrate in vial (Biomek 2000)
2. Collect 10 μl of whole blood into vial
3. Pipet 10 μl of internal standard in acetonitrile (Biomek 2000)
4. Pipet 50 μl of acetonitrile (Biomek 2000)
5. Recap and vortex
6. Centrifuge at 4000 rpm for 15 min
7. Transfer 75 μl of supernatant to clean vial (manual)

Calibration standards used a slightly modified protocol. Vials containing 20 μl of citrate buffer were supplemented with 10 μl of whole blood and 10 μl of the compound dissolved in citrate buffer at specific concentrations (0.1, 0.5, 1.0, 5.0, 10.0 and 100.0 μg/ml). The sample processing continued as per Table 1 (step 3) after this point. A standard curve was also generated using typical protein precipitation with acetonitrile. Plasma (100 μl) was treated with acetonitrile containing internal standard (90 μl) after addition of compound dissolved in acetonitrile at specific concentrations (10 μl). Standard blanks as well as double blanks were used to ensure the reliability of the quantitation. Calibration curves were generated using $1/x$ weighting of standards.

2.5. Liquid chromatography–mass spectrometry

Separations were performed on a Waters Alliance 2690 or 2790 LC system (Milford, MA, USA) controlled with MassLynx software or on a Perkin-Elmer Series 200 micropump LC and autosampler (Norwalk, CT, USA) controlled with MassChrom software. Samples (25 μl) were injected onto a Phenomenex Luna C_{18} column (50×2 mm, 5 μm particles) equipped with a SecurityGuard C_{18} guard. A steep gradient (10–90% acetonitrile vs. 20 m*M* ammonium acetate in 5% methanol in 2 min) at a flow-rate of 1.5 ml/min was used to elute the compound and internal standard. A post-column four-port valve (Valco) was used to divert the first 0.5 min of eluent to waste in order to minimize contamination of the ion source. The flow was split prior to entering the electrospray source such that ~300 μl/min entered the source with the remainder going to waste. Mass spectral data were generated with either a Micromass Quattro LC (Manchester, UK) or Sciex API-2000 (Concord, Canada) triple-quadrupole mass spectrometer using multiple-reaction-monitoring (0.2 s dwell per channel) for detection of the parent and internal standard. Data were acquired for 2 min with injections occurring every 3 min.

Typical operating conditions for the mass spectrometers were as follows:

Micromass Quattro LC: ion source: electrospray in the negative-ion mode; scan mode: MS–MS; scan type: multiple reaction monitoring (MRM), dwell time/channel 0.2 s. Nebulizing gas: 93 l/h; desolvation gas: 728 l/h; source block temperature: 120°C; desolvation temperature: 375°C.

Sciex API-2000: ion source: TurboIon spray in the negative-ion mode; scan mode: MS–MS; scan type: MRM, dwell time/channel 0.2 s. GS1: 40; GS2: 75; CUR: 25; CAD: 5; TEM: 400°C.

Collision energies used were compound dependent with 35 eV (laboratory frame of reference) being a typical value.

3. Results and discussion

Many murine models of human disease utilize mice that are transgenic, mutant or gene knockout and therefore are of limited availability and/or expensive. Routine PK screening in these mice can be prohibitive because of the large number of animals required and this prompted the investigation of using small volumes of blood from serially bled mice for PK studies. Several factors had to be addressed including the method of sample collection, processing and analysis. Dealing with whole blood can be problematic in terms of clotting and the quantitative transfer of samples. The processing method should entail a minimum number of steps with a maximum amount of automation to ensure reliability; and the analysis must be robust, fast and sensitive to maximize throughput.

Minimizing the number of sample transfers is a key requirement for maximum sample recovery when dealing with small volumes. Collection of the whole blood into a vial that could be used for sample processing would meet this requirement. However, coagulation of the blood can be problematic if the sample is not collected into a suitable buffer. Therefore, several different buffers/solvents were evaluated for collection of the whole blood with varying success. Citrate is commonly used to prevent coagulation of whole blood and was found to be suitable for sample collection. With the sample collected into a vial containing citrate, no coagulation was observed and sample processing (addition of internal standard and acetonitrile) could be carried out in the same vial. Addition of acetonitrile followed by vortexing resulted in protein precipitation. After centrifugation and careful transfer to clean inserts, the samples were clear and did not limit column lifetime. An important aspect to note is that the sample is being diluted by a factor of 10 (10 μl blood in 100 μl final volume) and for compounds that have low blood levels this may be problematic. However, since this method has been implemented no such case has arisen.

The analysis of samples prepared with this method required no modifications to the LC–MS protocol typically used when analyzing plasma samples. The resultant chromatography obtained for samples prepared with this protocol is shown in Fig. 1. Extracted ion chromatograms are shown for compound D (Fig.

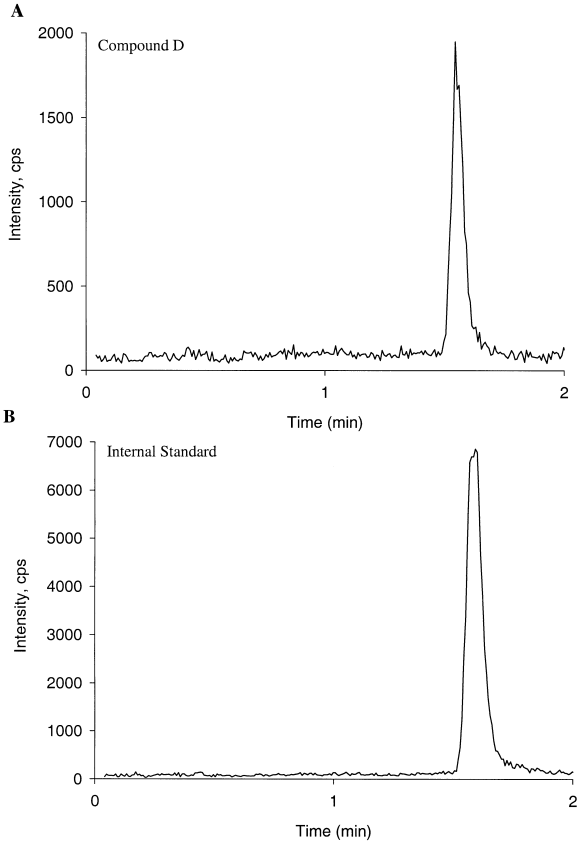

Fig. 1. Extracted ion chromatograms (MRM) for compound D (A) and the internal standard (B) at a concentration of 100 ng/ml (10 ng/ml effective) following a 25-μl injection onto a 2-mm column at a flow-rate of 1.5 ml/min under gradient conditions.

1A) and the internal standard (Fig. 1B). For both compounds the concentration injected was 100 ng/ml which is equivalent to 10 ng/ml after sample processing. Calibration curves were generated using the new protocol for comparison with the standard method of protein precipitation with acetonitrile (Fig. 2). The slope of the curve for the new protocol is approximately half that of the protein precipitation method. This is expected since the dilution factor if 10-fold versus twofold, and the final volume is 100 μl versus 200 μl, giving an expected difference of 2.5-fold in favor of the protein precipitation method. Typical column lifetime when using this method is approximately 300 injections with the guard column changed every ~100 injections.

An example of the pharmacokinetic data obtained using this method is shown in Fig. 3. For each curve, four animals were dosed requiring the use of 16 animals in total. Each curve represents the average of four animals at each of the nine time points, and the error bars are the standard error of the measurement. Using the conventional method of one mouse per

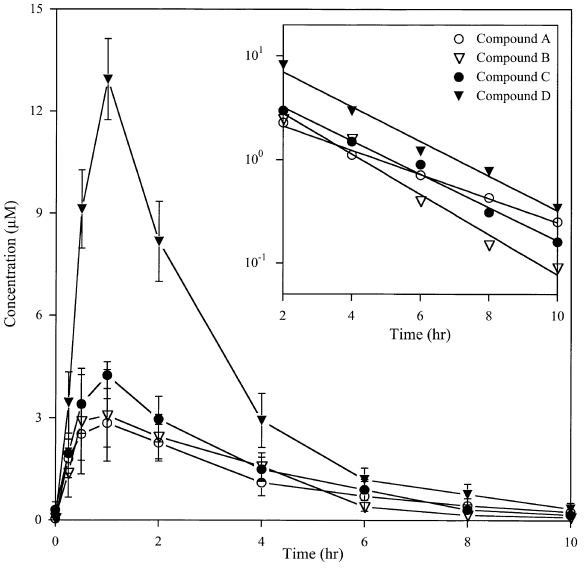

Fig. 3. Concentration versus time profiles for the 100 mg/kg oral administration to the mouse of four compounds for the benchmarking of a diet induced obesity model of diabetes. Inset shows same data plotted as semi-log plot.

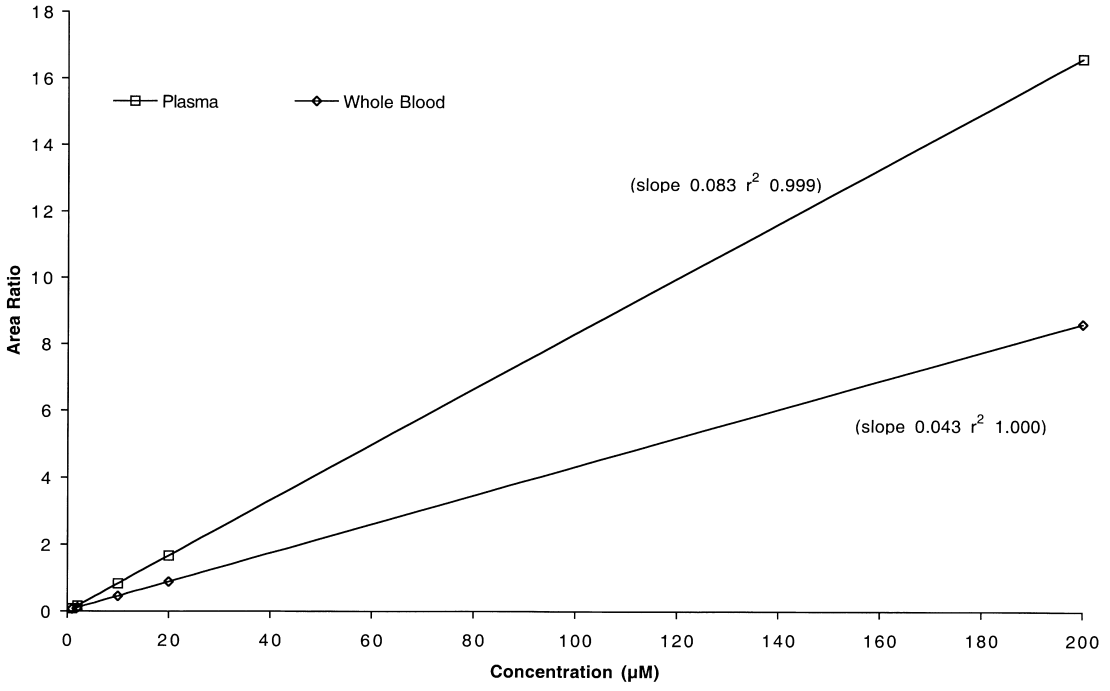

Fig. 2. Comparison of standard curves prepared using the new protocol versus the standard protocol of acetonitrile precipitation.

time point would have required 144 mice to generate similar data. Additionally, serially bleeding does not have the animal to animal variation associated with the one animal per time point method. Analysis of all samples plus standards required less than 8 h. In order to determine the terminal half-life of dosed compounds later time points are usually necessary. To this end the sampling interval can be modified to collect later time points if required. In this case, when the data is replotted on a semi-log scale (Fig. 3, inset) it is evident that the terminal elimination phase is reached after 2 h and that the $t_{1/2}$ ranges from 1.5 h (compound B) to 2.5 h (compound A), with compounds C and D having intermediate $t_{1/2}$ values (1.8 h).

Compound D was used for benchmarking an in vivo animal model for diet induced diabetes. Its effect on glucose lowering in the high fat diet-induced obesity model for diabetes is shown in Fig. 4A. Glucose levels were determined from serial bleeds of the mice at various time points post-dosing. In order to correlate blood levels of the drug with in vivo efficacy samples were also taken for PK measurement. Samples were taken before dosing and 1, 3 and 5 h post-dosing. The blood levels measured are plotted in Fig. 4B. This data represents true in vivo correlation of drug efficacy with blood levels in mice.

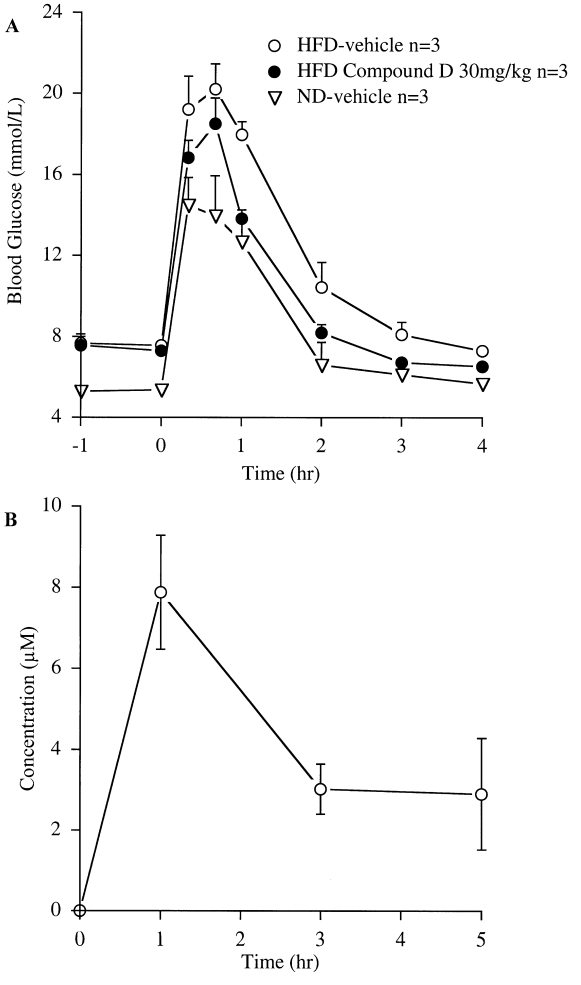

Fig. 4. (A) Effect of benchmarking compound D on the glucose tolerance of a mouse model of diabetes (HFD high-fat diet, ND normal diet). (B) Concentration versus time profile for compound D in the same mice used in the glucose tolerance test.

4. Conclusions

Serially bleeding of mice is an effective method for the reduction of animal usage for routine discovery stage PK studies. It also permits PD–PK correlation of in vivo efficacy of drugs with their blood levels in the same animal. Selection of the appropriate buffer for sample collection followed by semi-automated processing produces samples that can be analyzed without modification of existing analytical methods. Compounds administered at a low dosage or with low blood levels may not be detected because of the dilution, however this has not been a problem to date. This method has been used for over 200 studies to date for both routine PK studies and for studies of in vivo activity. This has reduced the use of a large number of animals. More importantly, rare and/or valuable mice used in pharmacological studies do not have to be euthanized and can be reused after a 3-week recovery and washout period.

References

[1] D. Malakoff, Science 288 (2000) 248.
[2] J. Ayrton, G.J. Dear, W.J. Leavens, D.N. Mallett, R.S. Plumb, J. Chromatogr. A 828 (1998) 199.
[3] D.A. McLoughlin, T.V. Olah, J.D. Gilbert, J. Pharm. Biomed. Anal. 15 (1997) 1893.

[4] D.A. McLoughlin, T.V. Olah, J.D. Ellis, L.D. Gilbert, R.A. Halpin, J. Chromatogr. A 726 (1996) 115.
[5] S. Steinborner, J. Henion, Anal. Chem. 71 (1999) 2340.
[6] A.P. Watt, D. Morrison, K.L. Locker, D.C. Evans, Anal. Chem. 72 (2000) 979.
[7] I.J. Fraser, G.J. Dear, R. Plumb, M. L'Affineur, D. Fraser, A.J. Skippen, Rapid. Commun. Mass Spectrom. 13 (1999) 2366.
[8] W.M.S. Russel, L.R. Burch, Principles of Humane Experimental Technique, Methuen, London, 1959.