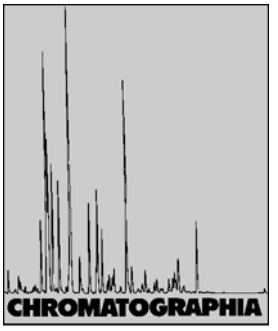
Serial Sampling in the Mouse in Support of Pharmacokinetic Studies with Turbulent Flow Chromatography and Tandem Mass Spectrometry



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Key Words

Column liquid chromatography
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On-line injection
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Summary

The bioavailability of a novel anticancer drug has been estimated in a cannulated mouse model, in which full pharmacokinetic profiles were obtained from a single animal. After withdrawing serial blood samples from the animal, small volumes of the plasma were prepared and analysed by the direct on-line injection of plasma into a turbulent flow chromatography extraction system linked to mass spectrometric detection. The use of these techniques significantly reduced both the number of animals needed to perform the experiment and the amount of active drug synthesised for administration whilst still maintaining adequate sensitivity to provide suitable pharmacokinetic profiles.

Introduction

Advances in proteomics, *in silico* molecular modelling and understanding of underlying biological mechanisms have facilitated the development of drugs that exhibit greater potency and efficacy whilst still being cytostatic and hence safer.

Often in the early discovery setting, the amount of pure drug available for testing has been limited. This has been particularly relevant when testing *in vivo* where clearly defined concentrations of active drug in the body have needed to be achieved for it to exhibit the desired effects. The amount of drug to be dosed is proportional to the body weight of the animal, hence, a mouse would require approximately 10 times less drug than a rat to achieve the same dose on a body weight basis.

Many xenograft tumour models have used the immune-deficient mouse strains [1]. While these are a convenient means of performing pharmacological tests on tumours *in situ*, they are time-consuming and it is costly to prepare the animals. Additionally, the models are only viable for a limited time due to excessive growth of the tumour if incubated for an inordinate period of time. Due to the small size of mice and the limited volume of blood that could be withdrawn [2], to construct a full pharmacokinetic (PK) profile required pooled sampling from several different mice, using traditional sample preparation techniques. However, it has been shown that small volumes of blood can be reliably taken over 24 hours whilst still maintaining the viability of the animal by use of an external jugular vein cannulation [3–6].

Conventional sample preparation techniques have tended not to provide sufficient sensitivity to allow measurement of drug levels 12 or 24 hours after drug administration when faced with limited sample volume unless microbore or capillary HPLC systems are used, coupled to mass spectrometry [7]. Techniques most amenable to this are those able to present at least a theoretical 100% of the extracted sample to the analytical instrument, and not a percentage, as has often been the case with extraction techniques such as solid phase extraction (SPE), liquid-liquid extraction (L-L) or plasma protein precipitation (PPP).

Recent publications have shown that direct, on-line injections of plasma in to a turbulent flow chromatography extraction system [8–14] coupled to a conventional analytical column operated under laminar flow conditions provides adequate separation of the drug from endogenous artefacts. An alternative form of on-line extraction [15] utilised column

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Table I. Pump timetable for the turbulent flow extraction system

Time (s)	Binary pump			Isocratic pump		
	Flow rate ($mL\,min^{-1}$)	Gradient type	ACN%	Flow rate ($mL\,min^{-1}$)	Channel	Tee
0	1	step	5	5	A	–
30	1	step	5	5	A	–
60	1	step	5	0.2	B	T
150	1	ramp	95	5	B	–
30	1	step	95	5	B	–
60	1	step	5	5	A	–

switching in conjunction with specialised columns or disposable cartridges operated under laminar flow conditions, though such systems have shown to suffer from carry-over limiting the dynamic range of the assay and are often complex in nature. The higher efficiency of the turbulent flow system reduces this effect in addition to shortening the cycle time of the procedure due to the much increased flow rates of the extraction column allowing rapid solvent changes and equilibration.

This study has demonstrated the application of turbulent flow chromatography to the analysis of very low plasma volumes whilst still maintaining adequate sensitivity. The cannulated mouse model has made it possible to reduce the number of animals needed, saving both valuable time and money whilst still providing a full PK profile. A bioavailability study was carried out where individual mice were used to obtain full PK profiles after oral and *intra venous* (IV) administration. This methodology has been applied to a series of compounds emerging from a novel Pharmacia anticancer discovery program. The chemical structure of the drug referred to in this publication cannot be disclosed for commercial reasons, however it was a basic molecule with an estimated lipophilicity of log *P* 3.1.

Experimental

Apparatus

The turbulent flow system consisted of a 2300 HTLC (Cohesive Technologies, Milton Keynes, UK) incorporating binary and isocratic pumps to give high and low pressure mixing respectively. Two Valco, 6-port switching valves were used along with a thermostatically controlled column-heating oven, used to house the analytical column. For the turbulent flow extraction step, a 50 mm × 1 mm i. d. column, containing 50 µm Polar Plus (Cohesive Technologies) and a Zorbax Rapid Analysis 75 mm × 4.6 mm i. d., 3.5 µm C8 analytical column (CPS, Italy) was used to perform the chromatographic separation prior to detection. The isocratic pump delivered 10 mM ammonium formate buffer, pH 3.5 – acetonitrile (95:5 *v/v*), at a flow rate of $5\,mL\,min^{-1}$ to the extraction column during the loading and washing steps. After 60 seconds of washing the proteins and endogenous species to waste, the drug was eluted from the extraction column, again by the isocratic pump, onto the analytical column at a flow rate of $0.2\,mL\,min^{-1}$ with 100% acetonitrile for 90 seconds by inserting the injection loop filled with 100% acetonitrile from the previous injection cycle into the flow path before the extraction column and teeing into the flow going to the analytical column. In this way, a total flow rate of $1.2\,mL\,min^{-1}$ was entering the analytical column during this step. During the time from the start of the cycle 10 mM ammonium formate buffer, pH 3.5:acetonitrile (95:5 *v/v*) as used initially was delivered to the analytical column by the binary pump at a flow rate of $1\,mL\,min^{-1}$. After the tee step, the isocratic pump increased and maintained the flow of acetonitrile at $5\,mL\,min^{-1}$ for a further 180 seconds to wash the extraction column and tubing whilst the binary pump initiated the gradient elution as described in Table I. On completion of the gradient elution of the drug from the analytical column, the system was re-equilibrated by the isocratic and binary pumps returning to the initial conditions. The complete cycle was performed in 5.5 minutes, after which the next plasma sample was injected. Injection of the plasma was performed by a HTC PAL (CTC Analytics AG, Switzerland) using a 10 µL Hamilton syringe and equipped with a Peltier cooler to reduce the sample storage compartment to a nominal 4 °C. The 2300 HTLC and HTC PAL were controlled by proprietary software supplied by the respective manufacturers.

Mass Spectrometer

An Applied Biosystems (Toronto, Canada) API365 triple quadrupole mass spectrometer controlled by Analyst 1.1 software was used. The instrument was operated in the positive ion mode using the heated nebuliser ion source. Data was collected from single reaction monitoring (SRM) scans, with a dwell time of 400 ms. All instrumental parameters were optimised to maximise the signal of the most abundant product ion after collisionally induced dissociation (CID) of the molecular ion in the second quadrupole.

Mouse Cannulation

CD-1 mice weighing 25–30 g were used (Charles River, Italy). After anaesthesia the animal was draped and prepared for aseptic surgery. To aid the surgical procedure, a stereomicroscope was used during surgery. The right external jugular vein was approached through a 1.0 cm ventral paramedian skin incision and careful blunt dissection. A 6 cm length of silastic catheter (105 or 135, Cow Dorning, USA), filled with heparinized saline solution, was gently inserted into the vessel to reach the right cardiac atrium. The catheter was then secured with two ligatures (silk 6/0) and placed around the vessel. The mice were kept in an incubator at 37 °C during recovery from anaesthesia and then allowed to recover for 3–5 days prior to experimental use. A daily check of both body weight and patency of the catheter was performed after the surgery. Blood samples were collected using a heparinized micro syringe. At each experimental time 50–70 µL of blood was drawn which amounted to a total of about 250–300 µL of blood in 24 hours. Using IV and oral routes, the doses ($5\,mg\,kg^{-1}$) were administered in the tail vein and by oral gavage with a single bolus, respectively. The blood samples were then immediately spun to obtain the plasma and then frozen at –80 °C ready for analysis.

Sample Preparation

Plasma samples were defrosted, vortex mixed and then centrifuged to remove any precipitated solids. The samples were then transferred from sample tubes to a 96-deep-well plate and placed in the autosampler ready for injection.

Results and Discussion

In total, six mice were successfully cannulated, three mice each for the IV and oral administration routes. Due to the small body size, surgery was a delicate procedure and some difficulty was experienced in some cases when advancing the catheter at the level of the thoracic inlet. Withdrawing and rotating the catheter and retrying helped alleviate this.

Mass spectrometric conditions were optimised to maximise the signal of the most abundant product ion. For this, the atmospheric pressure chemical ionisation (APCI) source was chosen, which gave a strong signal for the positive (pseudo) molecular ion with low background noise and performed optimally at the analytical column eluent flow rates of 1 mL min^{-1} as those used in the turbulent flow chromatography system. A SRM transition of *m/z* 339 > *m/z* 188 was chosen with a dwell time of 400 ms to ensure an adequate number of data points were acquired to accurately define the chromatographic peak. Both the first and third quadruples were operated at unit mass resolution, equivalent to a peak width of 0.7 amu at half height.

The turbulent flow chromatography system used a generic method developed in-house to support discovery projects. The risks of interference and ion suppression, particularly seen in unvalidated assays used to support discovery projects, had been minimised by use of the dual column approach. This mode of operation was more complex than utilising the extraction column alone (single column mode) to perform both extraction and chromatography roles in a very short cycle time, there being both an extraction column and a separate conventional analytical column to provide adequate resolution and good peak shape. During the development of the extraction method, each step of the pump programs and the timing of the switching valves were closely examined to find the optimal conditions to maximise recovery of the analyte of interest whilst minimising undesirable artefacts such as carryover and system pressure build-up. Of the steps, two were found to be particularly critical: the time and flow rate used during the transfer of the analyte from the extraction to the analytical column and the gradient conditions of the analytical column. When the flow rate and/or time used during the transfer step were too low, recovery was drastically reduced. This was due to incomplete elution of the drug from the extraction column, and was directly affected by changes in the system dead volume when the fluid pathways were modified in any way. The gradient elution used with the analytical column was critical as this had a profound effect on both the peak shape and the degree of interference in the form of a raised background. If the gradient parameters were timed correctly, the raised baseline would occur after the elution of the drug and thus had no effect on the assay. No problems were encountered with blocking or plugging the system with insoluble particulate matter, the system backpressure remaining stable over at least 200 injections.

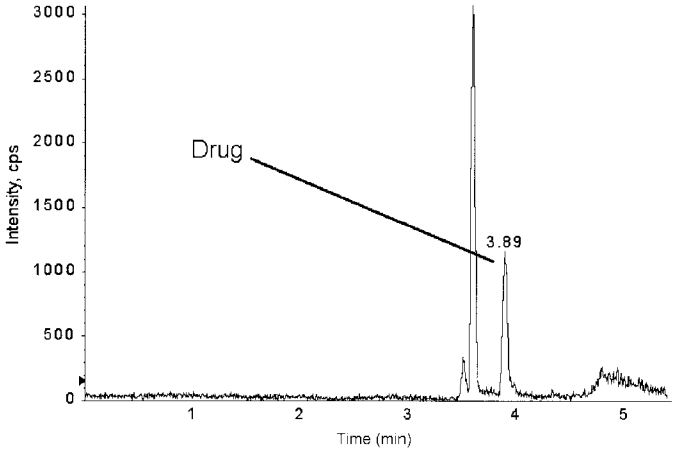

Figure 1. Mass chromatogram of a control plasma sample spiked with drug at a concentration of 1.6 ng mL^{-1}.

A lower limit of quantitation (LLOQ) of 1.6 ng mL^{-1} was achieved using a 2 µL injection of plasma, and is shown in Figure 1. This equated to approximately 3 pg of drug injected; the sensitivity assisted by the gradient elution of the analyte from the analytical column affording a narrow and symmetrical peak width of approximately 10 seconds. Two endogenous peaks could be seen at approximately 3.5 and 3.6 minutes. These peaks remained resolved throughout the assay and did not interfere with detection of the peak of interest. Carry-over was calculated to be approximately 0.15% by comparing the peak area of a plasma blank injected immediately after the upper limit of quantitation (ULOQ) Q^c. Whilst this would not be acceptable for a validated method, to support discovery it was considered adequate, and could be circumvented to a great degree by the insertion of additional blank plasma samples throughout the assay. The source of carry-over was determined to originate primarily from the switching valves. Calibration curves were shown to be linear over the nominal concentration range of 1.6–4500 ng mL^{-1} using a weighting factor of 1/x*x. Reproducibility of the assay was demonstrated by assaying duplicate calibration curves at the start and end of the assay. Though the assay did not use any form of internal standard, the curves were always super-imposable, suggesting recovery was adequately consistent across the concentration range analysed. A typical PK profile for both oral and IV treatments is shown in Figure 2.

Conclusion

The cannulated mouse model was shown to be suitable for use as a means of estimating oral bioavailability in the early drug discovery phase both conveniently and cost-effectively. Though very limited plasma volumes were sampled, a LLOQ of less than 2 ng mL^{-1} was achieved, allowing the reasonable definition of the PK parameters in the mouse. This provided a useful insight and link to oncology efficacy studies that commonly use xenograft mouse models, without the need for many animals. This analytical technique could also be applied to later-phase studies during a drug's development where limited sample is available as in paediatric studies. The turbulent flow chromatography proved to be a robust and sensitive methodology using conventional HPLC equipment.

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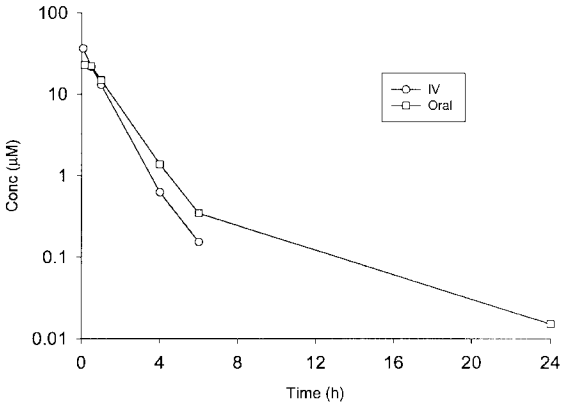

Figure 2. Typical pharmacokinetic profiles obtained after oral and IV dosing in the mouse.

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