Abstract

Background

The pharmacokinetic (PK) parameters in animal models can help optimise novel candidate drugs prior to testing them in man. However, due to the complexity of PK experiments, their use is limited in academia. We present a novel surgical rat model for investigation of the PK parameters and its use in an anti-obesity drug development program.

Materials and Methods

The model uses anesthetised male Wistar rats, a jugular and a femoral catheter and an insulin pump for peptide infusion. The following PK parameters were measured; metabolic clearance rate (MCR), half-life, and volume of distribution (Vd). Glucagon-like peptide 1 (GLP-1), glucagon (GCG) and exendin-4 (Ex-4) were used to validate the model. The PK parameters of anti-obesity drug candidates X1, X2 and X3 were then measured.

Results

GLP-1 had a significantly higher MCR (83.9±14.1 ml/min/kg) compared to GCG (40.7±14.3 ml/min/kg) and Ex-4 (10.1±2.5 ml/min/kg) ($p<0.01$ and $p<0.001$ respectively). Ex-4 had a statistically significant longer half-life (35.1±7.4 min) compared to both GCG (3.2±1.7 min) and GLP-1 (1.2±0.4 min) ($p<0.01$ for both GCG and GLP-1). Ex-4 had a statistically significant higher Vd (429.7±164.9 ml/kg) compared to both GCG (146.8±49.6 ml/kg) and GLP-1 (149.7±53.5 ml/kg) ($p<0.01$ for both GCG and GLP-1). Peptide X3 had a statistically significant longer half-life (21.3±3.5 min) compared to both X1 (3.9±0.4 min) and X2 (16.1±2.8 min) ($p<0.001$ for both X1 and X2).

Conclusions

We present an affordable and easily accessible platform for the measurement of the PK parameters of peptides. This novel surgical rat model produces consistent and reproducible results while minimising animal use.

Introduction

The development of high throughput approaches to drug development studies has been driven by the advances in high-speed chemistry and pharmacological screening [1]. There is a constant need for screening more and more compounds as the safety precautions for new drugs and the market competition increase [2, 3]. Pharmacokinetics involves investigating the mechanism by which a substance is absorbed and distributed within the body, any metabolism of the substance within the body, and finally the effects and the routes of excretion of the substance and its metabolites from the body [4-6].

In the drug development setting, PK parameters [half-life, metabolic clearance rate (MCR), volume of distribution (Vd)] are important as they allow characterisation of the relationship between drug dosage regimens and drug concentration-time profiles [7]. Over the last two decades, the use of PK modelling techniques to aid investigating the drug actions has become standard practice [8, 9]. Rodents, and in particular rats, are extensively used as experimental models for PK studies helping to optimise and select drugs prior to human trials [8, 10-15].

Drug discovery is extremely expensive with overall failure rates exceeding 80% while in the anti-obesity field the failure rates approach 99% [16-18]. Inadequate PK profiles and poor bioavailability of drug candidates are responsible for 10% of drug failures [19]. Pharmacokinetic experiments incur a significant cost (cost of the specialist equipment and consumables, purchasing and maintenance of animals, manpower and samples analysis cost) and these cost implications prevent many groups working on translational research from conducting PK studies during the discovery stages of drug development

An affordable PK animal model could significantly promote the use of PK early in drug development and potentially lead to a higher drug development success rate. As part of an anti-obesity drug discovery program we have successfully developed a surgical rat model for investigating the PK parameters of candidate drugs. The model includes cannulation of the right internal jugular vein, the left femoral vein and the use of a widely available, inexpensive, insulin pump for the intravenous (i.v.) infusion. In this study we present a surgical rat model for measuring the PK parameters of peptide drugs, its validation and the results obtained through its use.

Materials and Methods

The surgical rat model was used to measure the PK parameters of six peptides. Peptides GLP-1, GCG and Ex-4 were used to validate the model. Peptides X1, X2 and X3 were developed and tested as potential anti-obesity drug candidates.

Peptides

GLP-1, GCG and Ex-4 were purchased from Bachem, Ltd (Merseyside, UK) while peptides X1, X2 and X3 were synthesised by and purchased from Insight, Ltd (Wembley, UK). Peptides were synthesised using an automated fluorenylmethyloxycarbonyl solid phase peptide synthesis method with each amino acid sequentially added from the C to the N terminus. Peptides were cleaved from the resin then purified using reverse phase preparative high performance liquid chromatography (HPLC) followed by lyophilisation. All peptides supplied had a purity of >95% evaluated by HPLC.

Animal studies

All animal procedures undertaken were approved by the British Home Office under the UK Animal (Scientific Procedures) Act 1986 (Project Licence 70/7236). Adult

male Wistar rats (Charles River Ltd., Margate, Kent, UK) were maintained in double-housed cages under controlled temperature (21-23^{o}C) and light-dark cycles (12:12 hour light-dark schedule, lights on at 0700). Animals were allowed ad libitum access to water and ad libitum access to RM1 diet (Special Diet Services) unless otherwise stated. To minimise any non-specific stress effects animals were regularly handled to allow acclimatisation.

Experiments

The study design is shown in Figure 1. Animals were anesthetised with isoflurane, a catheter was inserted in the right jugular vein and a baseline sample was taken. The peptide to be tested (GLP-1, GCG or Ex-4) was reconstituted at a concentration of 30nmol/mL, the pump and tubing loaded and primed with the peptide solution, prior to catheterising the left femoral vein. The pump was then connected to the femoral catheter and set at a flow rate of 0.3 ml/h and ran for 60 minutes. A single bolus dose of 1.5 nmol was administered just before the infusion only for Ex-4. Sampling through the jugular access was performed throughout the infusion and for 40 minutes post-infusion. Four animals were used for each peptide PK experiment.

Equipment

The pump used in this study was a Medtronic 407c insulin pump with a 1.0 ml reservoir (MMT-103A). The catheters used were polyethylene tubing (internal diameter (ID) 0.46 mm, outside diameter (OD) 0.91 mm) (Instech Solomon) [20].

Catheter preparation

All cannula tubing required was prepared before the start of the procedure. The catheter was cut to 12 and 10 cm length for the jugular and the femoral vein respectively. One end had a 1ml syringe with a 25 gauge needle attached to it. A 1ml syringe was filled with heparinised saline (100 I.U/ml) and attached to the needle. The

other end of the tube was cut with a scalpel blade to create a bevelled edge. Catheter implantation was performed with the help of surgical loupes with a magnifying force of 2.5-3.5x.

Pump and peptide preparation

Peptides were reconstituted in gelofusine (at a concentration of 30 nmol/mL) after the jugular catheter was inserted. The infusion pump was loaded and allowed to run for 20 minutes in order to allow the peptide solution to fill the femoral catheter. Intravenous infusion lasted for 60 minutes with an infusion rate of 0.3 ml/h.

Pump infusate concentration measurement

After the removal of the femoral catheter from the femoral vein, the pump was allowed to run inside a microtube for 20 minutes and the infusate was collected and assayed by radioimmunoassay (RIA). Pump infusate concentration was used to calculate the total amount of peptide infused over 60 minutes.

Sampling and hormone assays

A baseline sample of 500 μl was taken following the insertion of a jugular vein cannulation. A total of 19 other sample were taken at the following time points from the beginning of peptide infusion: 0, 30, 35, 40, 45, 50, 55, 60, 62, 64, 66, 68, 70, 75, 80, 85, 90, 95 and 100 minutes (Figure 1).

Blood samples were collected in 1.5 ml heparinized microtubes containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA, P2714). The tubes were then spun in a centrifuge (3-18K SciQuip, Sigma, USA) for 10 minutes at 3000g at 4^{o}C. The plasma supernatant was collected and stored at -80^{o}C until assayed. Measurements of peptides concentration were carried out by established in-house RIA and all samples were tested in duplicate [21, 22].

Anaesthesia

Animals were anaesthetised with gas anaesthetic (isoflurane via cone mask at 2%) with an appropriate gas scavenging system as previously described [23].

Jugular and femoral vein catheterisation

Jugular and femoral vein catheterisation were performed as previously described [23].

Calculations

The PK properties that were investigated and calculated for each of the peptides included the half-life, the MCR and the Vd. All three peptides were tested at a concentration of 30 nmol/mL and at an infusion rate of 0.3 ml/h. Plasma peptide concentration was measured for 100 minutes after termination of the peptide infusion. Steady state concentration was calculated as the mean of the five values obtained at 40, 45, 50, 55 and 60 minutes of the infusion phase. Peptide concentrations were plotted on a semi-logarithmic scale versus time and computed to yield the slope from which the half-life ($t_{1/2}$) was determined.

Plasma half-life was calculated as follows:

$t_{1/2}(\text{min}) = \ln(2) / \text{gradient}$ where ln(2) is the natural logarithm of 2 (0.693).

The metabolic clearance rate (MCR) was calculated using Tait's formula:

$\text{MCR}(\text{ml kg-1 min-1}) \equiv \text{infusion rate} / \text{steady state plasma concentration}$

Volume of distribution (Vd) was calculated using following formula:

$V_d \equiv t_{1/2} \times MCR / \ln(2)$ where ln(2) is the natural logarithm of 2 (0.693).

Statistical analysis

All data are expressed as the mean value ± SD. Comparisons of the PK parameters between the groups were performed using one-way analysis of variation (ANOVA) with Bonferroni's post-hoc test. In all cases, values of $p<0.05$ were considered

statistically significant. The following program was used for statistical analysis: (GraphPad Prism version 5.00 for Windows; GraphPad Software, San Diego, CA).

Results

The PK parameters of the peptides are shown in Table 1. The pre-infusion values (baseline levels) of GCG and GLP-1, were approximately 43 and 35 pmol/l respectively. The time-courses of the mean plasma concentrations of Ex-4, GLP-1 and GCG are shown in Figure 2 and those of X1, X2 and X3 are shown in Figure 3. Steady-state concentration levels were observed between 30 and 60 minutes after the start of the peptide infusion for all peptides. The steady-state concentration levels of Ex-4, GLP-1 and GCG were 16.8±4.2, 2.6±0.4 and 8.4±4.2 pmol/ml respectively (Table 1). Peptide X1 presented the highest steady-state concentration among all peptides tested in this study (28.7±13.6 pmol/ml).

GLP-1 had a significantly higher MCR (83.9±14.1 ml/min/kg) compared to GCG (40.7±14.3 ml/min/kg) and Ex-4 (10.1±2.5 ml/min/kg) ($p<0.01$ and $p<0.001$ respectively) (Figure 2). GCG had a significantly higher MCR than Ex-4 ($p<0.01$).

Ex-4 had a statistically significant longer half-life (35.1±7.4 minutes) compared to both GCG (3.2±1.7 minutes) and GLP-1 (1.2±0.4 minutes) ($p<0.01$ for both GCG and GLP-1).

Ex-4 had a statistically significant higher Vd (429.7±164.9 ml/kg) compared to both GCG (146.8±49.6 ml/kg) and GLP-1 (149.7±53.5 ml/kg) ($p<0.01$ for both GCG and GLP-1).

Peptide X3 had a statistically significant longer half-life (21.3±3.5 minutes) compared to both X1 (3.9±0.4 minutes) and X2 (16.1±2.8 minutes) ($p<0.001$ for both X1 and X2) (Figure 3).

Discussion

The surgical rat model presented in this study was developed and used as part of an anti-obesity drug development program in an academic setting. Three different peptides (GLP-1, GCG and Ex-4) were used to validate the model by measuring their PK parameters and comparing the results to previous published studies. Following results validation, the model was used to measure the PK parameters of 3 novel anti-obesity drug candidates for humans. The rat model shown here has been used to characterise the PK parameters of more than 50 drug candidates in >200 rats in our experience, producing consistent and reliable results (data not shown due to intellectual property restraints).

The PK properties of drug candidates are important for the designing of new and improved agents and they can only be calculated by in vivo experiments in animal models. Following validation of our rat model, we measured the PK parameters of drug candidates X1, X2 and X3.

The validation of our model was centred on being able to measure the PK parameters of native peptide hormones. The three peptides used, GCG, GLP-1 and Ex-4, were selected due to the widely different PK parameters that they present and their relevance to the target drug candidate of our anti-obesity drug discovery program.

The PK parameters of GCG, GLP-1 and Ex-4 as reported by this rat model fall well within what has been previously reported [10, 24, 25]. The half-life of GLP-1 reported by Oshima et al. in 1988 (39.5 min) is considered to be outside normal limits; the potential sources of error of that study include the frequent sampling resulting in cardiovascular instability (five samples, 1 ml each, were collected within a period of 10 min and after centrifugation the suspension of red cells was reinfused into the animal) [21].

The selection of the time-points and volumes of sampling in our paradigm allows for consistent and reproducible measurement of the PK parameters while minimising blood loss and cardiovascular effects. We have previously reported that the total mortality rate in our experience was 0.5% (2/200 rats) [23].

As the field of drug development keeps expanding and the importance of PK studies has become more widely accepted, reports of serial blood sampling and analysis for drug and/or drug metabolites have appeared in the literature with increasing frequency [26]. Regulatory requirements for accurate PK profiles of novel compounds is driving forward the development and refinement of methodology for determining plasma time course data for drugs and/or drug metabolites in rodents.

The development of an animal model for investigating the PK properties of drug candidates in a drug development program is a complex task and requires multiple critical decisions to be made including the species/strain of animals that is representative of human, the i.v. routes that best serve the PK properties that are investigated, the number and time-points of obtaining the blood samples, the compound dose, the duration/rate of the infusion and the use of a reliable delivery system.

The basic concept underlying the presented surgical rat model is that there are two different intravenous (i.v.) routes required [27, 28]. The first route is used for infusing the drug with the help of a pump and the second one is required for blood sampling. Previously different routes of i.v. access have been used, the most common routes include the jugular vein, the femoral vein and artery, the saphenous vein and the carotid artery; each route has its own advantages and disadvantages (Table 2) [10, 11, 15, 25].

In our experience, the use of carotid arteries for sampling requires a higher degree of technical abilities and a longer learning curve. Furthermore, in case of encountering technical difficulties in catheter insertion, blood loss might be higher due to the increased pressure in the arterial system in comparison to the venous one. The saphenous vein presents the advantage of being close to the surface, thus reducing the amount of dissection to locate it, however its small size makes the catheterisation process a challenge.

The femoral vein, in the authors's view presents some important advantages. It is straightforward to locate as it is found in a bundle, together with the femoral artery and the femoral nerve. It has an adequate diameter to allow easy insertion of catheters and it can be exposed along its course so that more than one venotomies can be attempted if initial attempt fails. Of course, dissecting free the femoral vein from the femoral artery is challenging and great care should be taken not to damage the artery. The use of the jugular vein for sampling is one of the preferred methods due to the proximity of the jugular vein to the surface and its distance from any other major vessels.

A point worth noting, based on our experience, is that catheters should be secured with the use of more than one ties, in particular the ones used for sampling over the ones used for infusing and whenever multiple samples over a long period of time are to be taken. The use of a single tie to stabilise the catheter has quite often proved inadequate resulting in the dislodgement of the catheter and inability to continue taking samples. One or two extra ties are not time-consuming and provide much more reassurance for uninterrupted blood sampling.

Other equipment routinely used by the authors include a reusable cautery pen and magnifying loupes. The use of the cautery pen allows for easy and rapid hemostasis,

minimising blood loss and in experienced hands can also be used for dissection, minimising operating time. Surgical loupes have proved valuable especially while climbing the learning curve of femoral vein catheterisation but also afterwards and their magnification is of the essence whenever the first catheterisation attempt fails and the vessel collapses making the localisation of the venotomy site difficult.

The advantages presented by the use of the proposed animal model for evaluating the PK properties of peptides include the affordability of the pump setting (pump and reservoir) due to its low cost which bypasses the need for an expensive dedicated pump system. The rest of the consumables used are easily accessible and the setting of the procedure can be relatively easily reproduced. At the same time the design of the study has been optimised to measure the PK properties of gut hormones or analogues developed based on gut hormones. Although our model was optimised for measurement of anti-obesity drug candidates based on gut hormones, it can be used for any type of PK experiments.

A limitation of the current setting is the limited infusion rate that the insulin pump offers. Being originally designed to deliver small quantities of insulin in humans, the Medtronic 407c pump has a maximum rate of infusion of 0.35ml/h. This may pose limitations to researchers who wish a faster infusion rate.

In summary, the proposed rat model provides an affordable and easily accessible platform for measuring the PK properties of peptides outside the facilities of large pharmaceutical industries. The authors feel that the surgical rat model for the measurement of the PK parameters of peptides described in this study can be a useful tool that can produce consistent and reproducible results while minimising animal use.

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Table 1: Comparison of PK properties (metabolic clearance rate, half-life and volume of distribution) and of the steady-state concentration of glucagon, GLP-1, exendin-4, X1, X2 and X3 peptides, as measured in the surgical rat model (n=4).

	Glucagon		**GLP-1**		**Exendin-4**		**X1**		**X2**		**X3**	
	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)
Metabolic clearance rate (ml/min/kg)	40.7	(14.3)	83.9	(14.1)	10.1	(2.5)	13.9	(8.1)	6.2	(3.5)	5.4	(0.2)
Half-life (min)	3.2	(1.7)	1.2	(0.4)	35.1	(7.4)	3.9	(0.4)	16.1	(2.8)	21.3	(3.5)
Volume of distribution (ml/kg)	146.8	(49.6)	149.7	(53.5)	429.7	(164.9)	81.6	(70.7)	141.9	(43.6)	164.1	(31.3)
Steady state concentration (pmol/ml)	8.4	(4.2)	2.6	(0.4)	16.8	(4.2)	28.7	(13.6)	14.0	(4.2)	24.9	(0.9)

GLP-1: Glucagon-like peptide 1, SD: Standard deviation, Values are shown as mean ± SD.

Table 2

Table 2: Published literature of the PK parameters of exendin-4, glucagon and GLP-1 in rats.

Peptide	Authors/Date	Species	Weight (g)	Route	Duration	Dose	MCR	Half-life (min)	Vd	(Css) nM
Ex-4	Young et al./2001	Male Sprague-Dawley rats	350-370	Infusion: R. Saphenous V. Sampling: R.Femoral A.	180 min	0.05, 0.5, 5, and 50 nmol/h	0.5 nmol: 8.3±0.7 ml/min 5 nmol: 4.8±0.4 ml/min 50 nmol: 3.7±0.5 ml/min	0.5 nmol: 28±5 5 nmol: 40±5 50 nmol: 49±7	-	0.5 nmol: 1.1±0.1 5 nmol: 19±1.9 50 nmol: 262±60
GCG	Emmanouel et al./1978	Male Sprague-Dawley rats	250-350	Infusion: Jugular V. Sampling: Carotid A.		2-10 ng/min	31.8±1.2 ml/min/kg	-	-	-
	Kervran et al./1990	Male Wistar rats	250-280	Infusion: Saphenous V. Sampling: Carotid	45 min	0.56 nmol	36±5 ml/min/kg	1.9±0.1	0.1±0.01 L/kg	-
	Oshima et al./1988	Male Wistar rats	500-550	Infusion: Femoral V. Sampling: Jugular V.	60 min	2 ug	46.7±13.3 ml/min/kg	5.8±1.0	0.24± 0.07 L	-
GLP-1	Young et al./2001	Male Sprague-Dawley rats	350-370	Infusion: R. Saphenous V. Sampling: R.Femoral A.	180 min	0.05, 0.5, 5, and 50 nmol/h	0.5 nmol: 35±11 ml/min 5 nmol: 34±4 ml/min 50 nmol: 38±3 ml/min	0.5 nmol: 1.2±0.9 5 nmol: 0.9±0.6 50 nmol: 0.5±0.2	-	0.5 nmol: 0.32±0.06 5 nmol: 2.49±0.45 50 nmol: 20.53±2.94
	Oshima et al./1988	Male Wistar rats	500-550	Infusion: Femoral V. Sampling: Jugular v.	60 min	100 ug	18.6±8.6 ml/min/kg	39.5±15.5	0.5±0.1 L	-

Ex-4: exendin-4, GCG: glucagon, GLP-1: glucagon-like peptide 1, Css: Steady-state concentration, "-" signifies that the respective parameter was not reported.

Figure 1: Graphical representation of the study design of the surgical rat model in anesthetised male Wistar rats. Plasma sampling points are displayed in black circles over time in minutes.

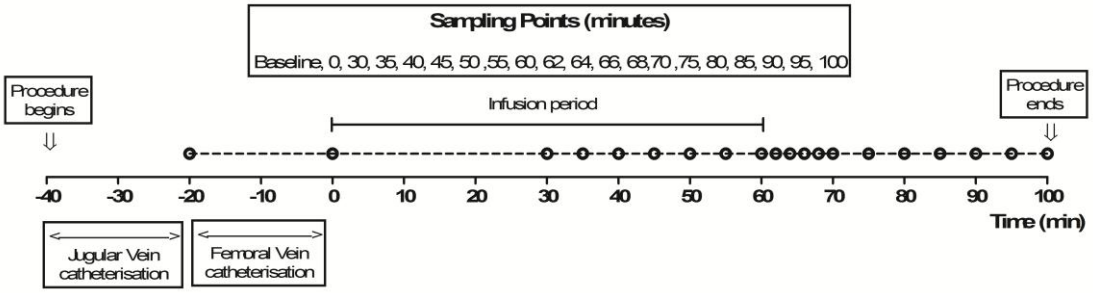

Figure 2: Plasma concentrations of glucagon, GLP-1 and exendin-4, and their pharmacokinetic parameters (metabolic clearance rate, half-life and volume of distribution) using the surgical rat model in anesthetised male Wistar rats. A: Semi-logarithmic plasma concentration of peptides over time, B: Metabolic clearance rate of peptides, C: Half-life of peptides and D: Volume of distribution of peptides. For all experiments n=4. Statistics with one-way ANOVA. ** =p<0.01, ***=p<0.001.

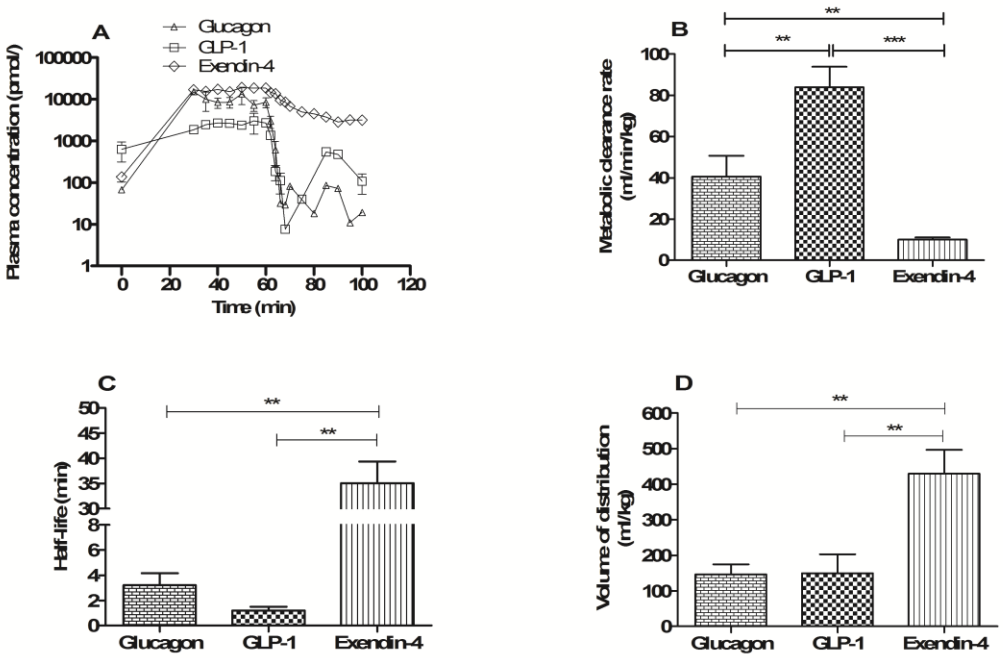

Figure 3: **Plasma concentrations of peptides X1, X2 and X3, and their pharmacokinetic parameters (metabolic clearance rate, half-life and volume of distribution) using the surgical rat model in anesthetised male Wistar rats. A: Semi-logarithmic plasma concentration of peptides over time, B: Metabolic clearance rate of peptides, C: Half-life of peptides and D: Volume of distribution of peptides. For all experiments n=4. Statistics with one-way ANOVA. ***=p<0.001.**