Research Article

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Bioanalysis

A sensitive method for the quantitation of the peptide-based glucagon-like peptide-1 receptor agonist liraglutide in plasma using microfluidics chromatography tandem MS

Amanda J King-Ahmad*[,1], Amit S Kalgutkar[2], Mark Niosi[1], Heather Eng[1] & Christopher Holliman[1]

[1]Medicine Design, Worldwide Research & Development, Pfizer, Inc., Eastern Point Road, Groton, CT 06340, USA

[2]Medicine Design, Worldwide Research & Development, Pfizer, Inc., 1 Portland St, Cambridge, MA 02139, USA

* Author for correspondence: Tel.: +1 860 686 2903; Amanda.King-Ahmad@Pfizer.com

Aim: An LC–MS/MS assay for the quantitation of liraglutide, a peptide-based injectable glucagon-like peptide-1 receptor agonist, has been developed as a convenient alternative to the enzyme-linked immunosorbent assay, and used to characterize liraglutide pharmacokinetics in cynomolgus monkeys. **Results:** Assay calibration curves exhibited a linear dynamic range of 10–5000 ng/ml and correlation coefficient ≥0.98. Following a 30 μg/kg intravenous dose, liraglutide demonstrated low plasma clearance and distribution volume, which led to a terminal half-life of 6.59 h in monkeys. **Conclusion:** The dynamic range of our LC–MS/MS assay provides sufficient coverage of the average efficacious liraglutide concentrations in human plasma, and can be used for pharmacokinetics/pharmacodynamics studies in animals and potentially in humans.

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Incretins are hormones that are released from endocrine cells in the small intestine into the bloodstream in response to oral nutrient ingestion [1,2]. Incretin hormones maintain glucose homeostasis by stimulating pancreatic β cells to release insulin in a glucose-dependent fashion. In healthy individuals, incretin effect accounts for up to 70% of the total insulin response to a meal [3]. Of the two incretins, namely glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide, GLP-1 has received more attention because it can increase insulin secretion and normalize blood glucose levels in patients with Type 2 diabetes mellitus (T2DM) [4–9]. GLP-1 is a 30-amino acid peptide (GLP-1(7–36)amide) hormone, which acts through a class B G protein-coupled receptor on pancreatic β cells to exert glucoregulatory and insulinotropic actions [8–10]. Following its secretion from the intestine, native GLP-1(7–36)amide is rapidly degraded (half-life ($t_{1/2}$) = 1–2 min) on its *N*-terminus by dipeptidyl peptidase-IV (DPP-IV) to yield GLP-1(9–36)amide as an inactive metabolite [11]. Because of the short circulating $t_{1/2}$ of native GLP-1, inhibitors of DPP-IV (e.g., sitagliptin) and DPP-IV-resistant GLP-1 receptor agonists have been developed as a treatment option for individuals with T2DM [12,13]. Liraglutide ($C_{172}H_{265}N_{43}O_{51}$, MW = 3751.202, Figure 1) is an injectable GLP-1 receptor agonist that has been recently approved for the treatment of T2DM (Victoza®) and weight management (Saxenda®) [14,15]. Liraglutide is an acylated GLP-1 analog, produced by recombinant DNA technology in *Saccharomyces cerevisiae*, in which Arg34 replaces Lys34 at the *N*-terminal and a fatty acid chain is added to Lys26 [16]. These changes, specifically the fatty acid chain addition, result in high protein binding to human plasma (>98.9%), and a substantially longer elimination $t_{1/2}$ (~10–18 h) relative to native GLP-1, which makes liraglutide suitable for once-daily administration [16–18].

Historically, quantitation of liraglutide in plasma to support pharmacokinetic and pharmacodynamic analysis has been performed using a proprietary ELISA [19]. The ELISA method measures total liraglutide plasma concentrations, is specific for intact liraglutide, and has an LLOQ of approximately 18 pM with a day-to-day variability of 3.7–10%.

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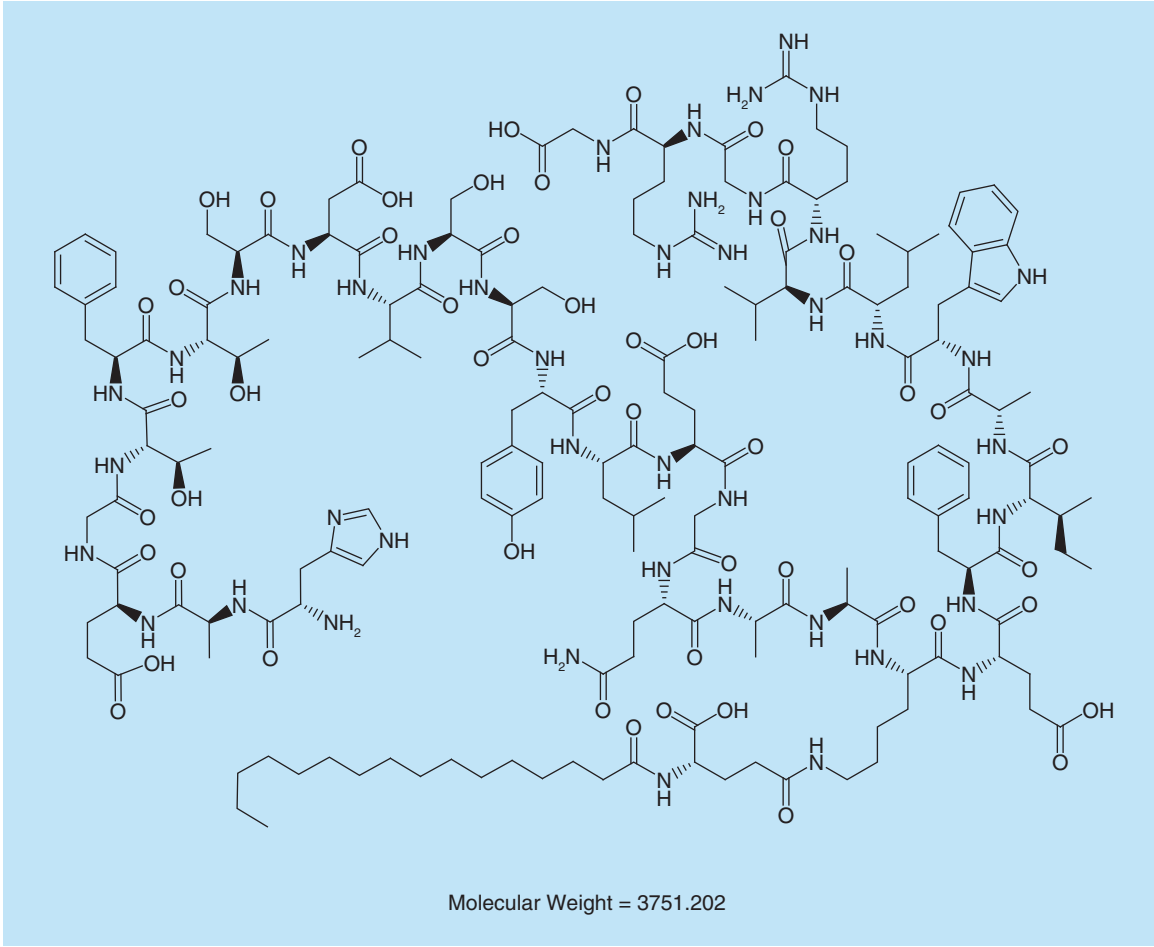

Figure 1. Chemical structure of liraglutide.

An attractive alternative to the ELISA technique is the potential for the detection and quantitation of liraglutide levels in biological matrices by LC–MS/MS. LC–MS/MS assays do not require the extensive method development work required to select appropriate antibodies, generally have a larger dynamic range and are more cost-effective. Nevertheless, LC–MS/MS assays can have their own challenges, especially when operating in the high MW range associated with peptide-based therapeutics. The high MWs of proteins and peptides can lead to multiple charge states of the parent ion and multiple fragmentation patterns resulting in decreased signal intensity and negative impact on the LLOQ of the assays [20,21]. Despite these caveats, sensitive LC–MS/MS quantitation methods in plasma have been reported for peptide-based agonists of the GLP-1 receptor [22,23], as well as active GLP-1(7–36)amide [24] and its inactive GLP-1(9–36)amide metabolite [24,25]. In this paper, we disclose our development and validation of a sensitive LC–MS/MS assay for the quantitation of liraglutide in plasma, which was also employed for the characterization of liraglutide intravenous (iv.) pharmacokinetic properties in cynomolgus monkeys, given our interest in exploring liraglutide pharmacology (glucose-dependent insulin stimulation and weight loss) in this species. The LC–MS/MS assay was developed using the Waters Corporation (MA, USA) I-Key separation tile (iKey), which integrates 150-µm inner diameter capillary chromatography with sub-10 µl/min flowrates and ESI in a readily accessible format [26]. The increases in ionization and ion transmission efficiencies that are gained by reducing the LC eluent flow to 3 µl/min enabled the development of a sensitive LC–MS/MS method that uses simple protein precipitation of a minimal quantity of monkey plasma, and produces a linear dynamic range of 10–5000 ng/ml (2.6–1330 nM). The dynamic range of this assay provides sufficient coverage of the average 24 h concentrations (~128 ng/ml, 34 nM) of liraglutide in human plasma at its efficacious dose of 1.8 mg for treatment of T2DM [27].

Experimental

Reagents & materials

For monkey pharmacokinetic studies, liraglutide was extracted from a Victoza Liraglutide Pen (lot #ES6P825, Novo Nordisk, NJ, USA) . Internal standard (IS) terfenadine and liraglutide (used in bioanalytical method development) were synthesized at Pfizer, Inc., CT, USA. Deionized water obtained from a Millipore water purification system (Millipore, MA, USA) was used for sample dilutions and LC mobile phase. Formic acid was purchased from Sigma-Aldrich (Sigma Aldrich Corporation, MO, USA). Ethanol, acetonitrile and all other chemicals and reagents were of analytical grade and purchased from commercial sources. Blank pooled cynomolgus monkey plasma was purchased from BioreclamationIVT (Westbury, NY, USA). Individual blank male cynomolgus monkey plasma was obtained in-house (Pfizer) from blood collected into K_2EDTA tubes. All animal care and *in vivo* procedures were conducted in accordance with the guidelines set by the Pfizer Institutional Animal Care and Use Committee.

LC–MS/MS conditions

Assay development, validation and sample analysis were performed using a Waters Acquity M-Class UPLC (Waters Corporation), and a Waters IonKey Xevo TQ-S (Waters Corporation). Chromatographic separation of the analyte was achieved on an iKey with a 150 µm × 50 mm channel packed with HSS T3 100A, 1.8 µm diameter particles (Waters Corporation) using a 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) gradient. The gradient consisted of an initial hold at 25% B for 0.1 min, a liner ramp to 98% B over 2.9 min, a second hold at 98% B for 0.2 min, a return to 25% B over 0.2 min, followed by a re-equilibration at 25% B for 1.6 min to give a total chromatographic run time of 5.0 min at a flow rate of 3.0 µl/min. Analyte and IS detection was achieved in positive ion mode with the following settings: capillary voltage = 3.5 kV, source temperature = 120°C and cone gas = 150 l/h. Ions were monitored via multiple reaction monitoring using the doubly charged parent of liraglutide and the Q1→ Q3 transitions of m/z 938.8 → m/z 1128.5 (cone voltage = 100 V, collision energy = 26 V) for liraglutide and m/z 472.6 → m/z 436.46 (cone voltage = 50 V, collision energy = 35 V) for the IS terfenadine.

Standard & quality control sample preparation

Two liraglutide stocks were prepared at 1 mg/ml in 50:50 acetonitrile:dimethyl sulfoxide and compared with confirming accurate weighing of the analyte. From one of these stocks, an intermediate 1 µg/ml stock was prepared in 50:50 acetonitrile:dimethyl sulfoxide. Standard samples were prepared by spiking pooled cynomolgus monkey plasma with the intermediate stock and serially diluting to produce plasma concentrations of 10, 25, 50, 100, 250, 500, 1000, 2500 and 5000 ng/ml. Quality control (QC) samples were prepared in the same manner at the concentrations of 10, 30, 230 and 4000 ng/ml, respectively, corresponding to the LLOQ QC, the low QC (LQC), the mid QC and the high QC (HQC). QC sample pools were divided into 1 ml aliquots, stored at -20°C and used throughout the validation. Plasma standards were prepared fresh daily. A single stock solution of terfenadine was prepared at 1 mg/ml in 50:50 acetonitrile:dimethyl sulfoxide and subsequently diluted to 30 ng/ml in ethanol to prepare the IS protein crash solution, which was stored at ambient temperature. Since the assay was primarily developed to support discovery pharmacology studies, radio or stable isotope-labeled liraglutide IS was not synthesized. Instead, terfenadine was selected as an IS based on its similar retention time under the chromatographic conditions and its availability in our laboratory.

Sample preparation

Monkey plasma samples were prepared by precipitation of plasma proteins from 50 µl of sample with 100 µl ethanol containing the IS terfenadine (30 ng/ml). Similarly, matrix double blank samples were prepared by precipitation of blank plasma with ethanol. The precipitated samples were vortexed for 1 min followed by centrifugation at 3000 r.p.m. (1643 × *g*) for 5 min. From these, 50 µl of supernatant was transferred to a clean 96-well plate, diluted with 100 µl of 0.1% formic acid in water and 5 µl of this final extract was analyzed by LC–MS/MS.

Assay validation

Calibration & linearity

Data were acquired and chromatographic peaks were integrated using Targetlynx software (Waters Corporation). Calibration curves were constructed using a linear regression with $1/x^2$ weighting and by plotting the plasma

standard concentrations verses the corresponding chromatographic peak area ratios of liraglutide over the IS terfenadine. The linear range of the calibration curve was 10–5000 ng/ml.

Accuracy & precision

Inter- and intraday accuracy and precision were established by analysis of six replicates (n = 6) of each of the four QC levels (10, 30, 230 and 4000 ng/ml), on three different days, quantitating against fresh standards daily.

Selectivity

Selectivity of the assay was evaluated using blank plasma from six individual cynomolgus monkeys. Blank (with IS) and double blank (no IS) samples were prepared from the individual plasmas and assessed for chromatographic interference at the retention times of the analyte and IS.

Plasma sample stability

Liraglutide stability in plasma was assessed at room temperature (benchtop stability), at -20°C (freezer stability), and through two and three freeze-thaw cycles using LQC and HQC samples. Large pools were prepared at LQC and HQC concentrations, were aliquoted and used for the stability assessments. Room temperature samples were placed on the benchtop for 24 h and then stored at -20°C until analysis. Freeze–thaw stability samples were subjected to up to three freeze–thaw cycles. Each cycle consisted of thawing for 2 h at room temperature, under normal laboratory light conditions, and then returning to the freezer for a minimum of 12 h. Long-term freezer stability samples were stored at -20°C for 14 days. For analysis, benchtop, long-term freezer and freeze–thaw samples were thawed, prepared as described above and analyzed by LC–MS/MS against freshly prepared standards to determine the plasma stability of liraglutide under the various conditions.

Extract stability

Extract stability was assessed after 12 days. Following analysis of the day 1 validation samples, the extracts were stored in the autosampler at 10°C for 12 days. The extracts were then reinjected and quantitated against freshly prepared standards.

Extraction solvent determination

Extraction efficiency of three solvents, acetonitrile, methanol and ethanol, was evaluated by the analysis of spiked plasma extracts and blank plasma extracts over spiked with liraglutide (spiked blank extracts), in triplicate. Spiked plasma extracts were prepared at a nominal concentration of 5000 ng/ml. Spiked plasma extracts were prepared by extracting 50 µl of spiked plasma with 100 µl of IS in ethanol, methanol or acetonitrile, as described above, and diluting 50 µl of the resulting supernatant with 100 µl of water containing 0.1% formic acid. Spiked blank extracts were prepared by extracting 50 µl of blank plasma with 100 µl of IS in ethanol, methanol or acetonitrile, as described previously, and diluting 50 µl of the resulting supernatant with 100 µl of water containing 0.1% formic acid and liraglutide (835.5 ng/ml). Analyte extraction efficiency from the matrix was determined for each solvent by comparing the peak area response of spiked plasma extracts to the peak area response of the spiked blank extracts.

Analyte extraction efficiency, matrix effects & compensation of the matrix effects by the IS

Analyte extraction efficiency, matrix effects and compensation of the matrix effects by the IS were determined by the analysis and comparison of the responses of spiked plasma extracts, blank plasma extracts which were over spiked with liraglutide (spiked blank extracts) and neat liraglutide solutions, in triplicate. Spiked plasma extracts were prepared at a nominal concentration of 5000 ng/ml and extracted as described. Blank plasma extracts were prepared by extracting 50 µl of blank plasma with 100 µl of IS in ethanol, as described above, and diluting 50 µl of the resulting supernatant with 100 µl of water containing 0.1% formic acid and liraglutide (835.5 ng/ml). Equivalent neat liraglutide solution was prepared by mixing 50 µl of water with 100 µl of IS in ethanol. Fifty microliters of this solution was then diluted with 100 µl of water containing 0.1% formic acid and liraglutide (835.5 ng/ml). All samples were analyzed by LC–MS/MS.

Analyte extraction efficiency (recovery), from the matrix was determined by comparing the peak area response of spiked plasma extracts to the peak area response of the spiked blank extracts. This comparison normalizes any loss of response due to matrix effects so that the difference between the two solutions is attributed to the analyte

Table 1. Summary of extraction efficiencies, matrix effects and internal standard compensation of matrix effects on liraglutide in cynomolgus monkey plasma.

Matrix	Peak area		Peak area ratio analyte/IS
	Mean lot-to-lot extraction efficiencies (%)	Mean lot-to-lot Δ response due to matrix effects (%)	Mean lot-to-lot Δ response matrix effect with IS (%)
Monkey 1 plasma (n = 3)	104	-78.9	-23.7
Monkey 2 plasma (n = 3)	97.6	-79.1	-25.1
Monkey 3 plasma (n = 3)	95.9	-78.4	-20.6
Monkey 4 plasma (n = 3)	96.9	-78.2	-19.4
Monkey 5 plasma (n = 3)	73.2	-82.8	-32.5
Monkey 6 plasma (n = 3)	77.7	-79.3	-9.55
Pooled monkey plasma (n = 3)	98.7	-82.5	-27.0
All determinations (n = 21)			
Mean	92.0	-79.4	-22.6
%CV	13.0	3.20	33.0

IS: Internal standard.

extraction efficiency. Changes in analyte response due to matrix effects were determined by comparing the peak area response from the neat liraglutide solution to the spiked blank extract. The difference in the observed peak area response is attributed to matrix ionization effects. The degree of response normalization by the addition of the IS was assessed by comparing the peak area response ratios of analyte/IS. Similar to the above assessment, changes in response due to matrix effects were determined by comparing the peak area response ratios from neat liraglutide solution to the spiked blank extract. The difference in the observed response ratio is attributed to incomplete or over compensation of matrix ionization effects by the IS.

Animal pharmacokinetics

Pharmacokinetic studies in male cynomolgus monkeys (n = 2) were conducted in-house at Pfizer. Liraglutide was extracted from a Victoza Liraglutide Pen (lot #ES6P825) and formulated in 1.35% (v/v) propylene glycol:98.65% (v/v) Dulbecco's phosphate-buffered saline, (pH = 7.4) and administered as an iv. bolus via the saphenous vein at a dose of 30 μg/kg. Serial blood samples were collected at the 0, 0.083, 0.25, 0.5, 1, 2, 4, 7 and 24-h time points via the femoral vein by syringe and transferred into K_3EDTA vacutainers. Blood samples were then centrifuged, the plasma harvested and stored at -20°C or -80°C until analysis. All animal care and *in vivo* procedures were conducted in accordance with the guidelines set by the Pfizer Institutional Animal Care and Use Committee.

Determination of pharmacokinetic parameters

The pharmacokinetic parameters for plasma clearance (CL_p), terminal elimination $t_{1/2}$ and volume of distribution (Vd_{ss}) were calculated from the iv. dose, using a noncompartmental analysis (Watson v.7.4, Thermo Scientific, MA, USA).

Results

Analyte extraction efficiency & determination of the protein precipitating solvent

The extraction efficiency of liraglutide from monkey plasma was evaluated using acetonitrile, methanol and ethanol as the protein precipitating solvent. Recovery in the aprotic solvent acetonitrile was low at approximately 30% whereas the protic solvents methanol and ethanol yielded higher liraglutide recoveries of 78 and 86%, respectively (data not shown). Extraction recovery of liraglutide from six individual and one pooled lot of cynomolgus monkey plasma was determined in triplicate using a 2:1 volume of ethanol:plasma. The observed mean recoveries from the lots ranged from 73.2 to 104% (Table 1).

Assessment of matrix ionization effects

The impact of the sample matrix on the ionization efficiency of liraglutide was evaluated using six individual and one pooled lots of cynomolgus monkey plasma. Each lot was assessed in triplicate. All lots evaluated demonstrated suppression of the liraglutide signal as measured by peak area relative to the response of an equivalent neat solution of liraglutide. The observed mean peak area suppression from the lots ranged from -82.8 to -78.2%, Table 1.

Table 2. Summary of inter- and intraday accuracy, and precision for liraglutide standards in cynomolgus monkey plasma.

Nominal concentration (ng/ml)	Standards (n = 1)			Standards (n = 3)	
	Accuracy			Interday	
	Day 1	Day 2	Day 3	Mean accuracy	%CV
10	103.7	99.6	109.4	104.2	4.7
25	91.9	104.9	82.2	93.0	12.2
50	95.0	92.9	85.5	91.1	5.4
100	107.9	100.9	106.7	105.2	3.6
250	88.6	93.6	95.7	92.7	3.9
500	110.9	100.4	101.0	104.1	5.7
1000	103.9	101.5	106.5	104.0	2.4
2500	93.1	104.1	101.5	99.5	5.8
5000	105.0	102.2	111.4	106.2	4.5

Table 3. Summary of inter- and intraday accuracy, and precision for liraglutide quality control samples in cynomolgus monkey plasma.

Nominal concentration (ng/ml)	QCs (n = 6)						QCs (n = 18)	
	Day 1		Day 2		Day 3		Interday	
	Accuracy	%CV	Accuracy	%CV	Accuracy	%CV	Mean accuracy	%CV
10	99.0	13.0	91.7	5.4	102.3	14.3	97.6	12.1
30	93.9	5.5	97.0	12.2	93.6	8.4	94.8	8.8
230	102.5	2.5	99.4	7.2	101.0	9.2	101.0	6.6
4000	105.2	1.1	101.5	2.5	113.2	3.7	106.6	5.4

QC: Quality control.

Assessment of matrix ionization effects compensation by the IS

The ability of the IS to normalize the liraglutide response was evaluated using six individual and one pooled lots of cynomolgus monkey plasma. Each lot was assessed in triplicate. All lots evaluated produced a lower peak area ratio as measured by the peak area ratio (analyte/IS) relative to the response of an equivalent neat solution of liraglutide and IS. The change in the mean peak area ratios ranged from -32.5 to -9.55% indicating that the IS only partially compensates for the matrix-induced variability of the liraglutide response. The observed mean percent change in the peak area ratios from the lots is presented in Table 1.

Assay validation

Validation batch runs consisted of a matrix blank and double blank, followed by a single set of liraglutide plasma standards split between the beginning and end of the run which bracketed six sets of the QC samples. The batch runs were prepared and analyzed on three separate days and a new set of liraglutide monkey plasma standards was prepared fresh the day of each run. Standard and QC accuracies were determined based on their nominal values. The batch runs and subsequent stability assessments were evaluated using the accuracy and precision acceptance criteria recommended by the US FDA and EMA [28,29].

Calibration & linearity

Calibration curves on all 3 days generated a linear regression coefficient (R^2) $\geq$0.98 when a $1/x^2$ weighting was used. Back-calculated standard accuracies over the three batch runs ranged from 82.2 to 111%. The interday accuracies and %CV values of the back-calculated standards ranged from 91.1 to 106% and 2.4 to 12.2%, respectively. A summary of the standards data is presented in Tables 2 & 3.

QC accuracy & precision

QC standards were used to assess the inter- and intraday accuracy and precisions of the assay. Nominal values for the QC samples were selected as follows. The LLOQ QC of 10 ng/ml (2.7 nM) was selected based on the LLOQ of the assay, the LQC of 30 ng/ml (7.9 nM) was selected at three-times the LLOQ of the assay, the mid QC of

Table 4. Summary of liraglutide stability in cynomolgus monkey plasma under various conditions.

Assessment	LLOQ QC (10 ng/ml)		LQC (30 ng/ml)		MQC (230 ng/ml)		HQC (4000 ng/ml)	
	Accuracy	%CV	Accuracy	%CV	Accuracy	%CV	Accuracy	%CV
24-h Benchtop (n = 6)			114.8	16.5			109.3	3.4
14 day long-term storage stability			107.1	5.8			91.0	9.4
3 Freeze–thaw cycle			112.0	10.5			98.2	12.1
Extract stability (12 day)	92.0	13.8	85.1	10.1	93.5	4.2	102.1	4.0

HQC: High quality control; LQC: Low quality control; MQC: Mid quality control; QC: Quality control.

Table 5. Intravenous[†] monkey pharmacokinetics parameters for liraglutide.

Dose (μg/kg)	CL_p (ml/min/kg)	V_{dss} (ml/kg)	$t_{1/2}$ (h)	$AUC_{(0-\infty)}$ (ng.hr/ml)
30	0.0447 (0.0518, 0.0376)	21.6 (21.7, 21.5)	6.59 (5.59, 7.58)	11500 (9650, 13300)

Male cynomolgus monkeys were utilized for pharmacokinetics studies.
All data reported here are means from n = 2 animals (individual values are depicted in parenthesis).
[†]Vehicle was 1.35% (v/v) propylene glycol:98.65% (v/v) Dulbecco's PBS (pH = 7.4).
CL_p: Plasma clearance; Vd_{ss}: Volume of distribution.

230 ng/ml (61.3 nM) was selected based on the geometric mean of the standards and the HQC of 4000 ng/ml (1066.3 nM) was selected based of 80% of the upper limit of quantitation. Mean accuracy and precision (%CV) at each QC level were determined on each day of validation and over the 3 validation days to determine the inter- and intraday accuracy and precision of the assay. The acceptable limits for the validation were ±15% for both mean accuracy of nominal concentration and %CV of both inter- and intraday analysis. As shown in Tables 2 & 3, the intraday accuracies (n = 6) of the QC samples ranged from 91.7 to 113% and the intraday %CVs ranged from 1.10 to 14.3%. The interday accuracies (n = 18) of the QC samples ranged from 94.8 to 107% and the interday %CVs ranged from 5.40 to 12.1%.

Monkey plasma stability

Plasma samples collected from *in vivo* pharmacokinetic (or pharmacodynamics) studies are typically not analyzed immediately; rather they are frozen and stored for analysis at a later time. Consequently, the stability of liraglutide in cynomolgus monkey plasma at both the LQC and HQC levels was examined under several storage conditions (Table 4). Following storage at each of the conditions tested, six replicates at each QC level were analyzed against fresh standards and all samples demonstrated accuracy ±15% from their nominal concentration (Table 4). Plasma extract stability of liraglutide was established for 12 days when stored at 10°C by reinjection of the QC extracts from the first batch run and quantitating against a set of freshly prepared liraglutide plasma standards. Reinjection and quantitation of the extract stability QC against fresh standards resulted in all concentrations being within ±15% of their nominal values. These studies established that liraglutide is stable in monkey plasma for at least 14 days at -20°C, and through at least three freeze–thaw cycles. Additionally, liraglutide was found to be stable in monkey plasma for at least 24 h on the bench top.

Selectivity

Assay selectivity was demonstrated from the analysis of six individual lots of monkey plasma. No distinct chromatographic interferences were observed at the retention time of liraglutide (3.8 min) or terfenadine (3.6 min) indicating that the assay is selective for the analytes of interest in monkey plasma. Representative chromatograms generated for a matrix blank and an LLOQ sample are shown in Figure 2. It is noteworthy to point out that in the liraglutide multiple reaction monitoring channel, an increase in baseline was observed between 3.0 and 4.5 min; however, no distinct peaks were observed at the retention time of liraglutide in blank samples (Figure 2C).

Monkey pharmacokinetics

The pharmacokinetic parameters describing the disposition of liraglutide after iv. bolus administration at 30 μg/kg to male cynomolgus monkeys are shown in Table 5 & Figure 3. Liraglutide demonstrated low CL_p (0.0447 ml/min/kg) and low Vd_{ss} (21.6 ml/kg) resulting in a terminal elimination $t_{1/2}$ of 6.59 h in monkeys.

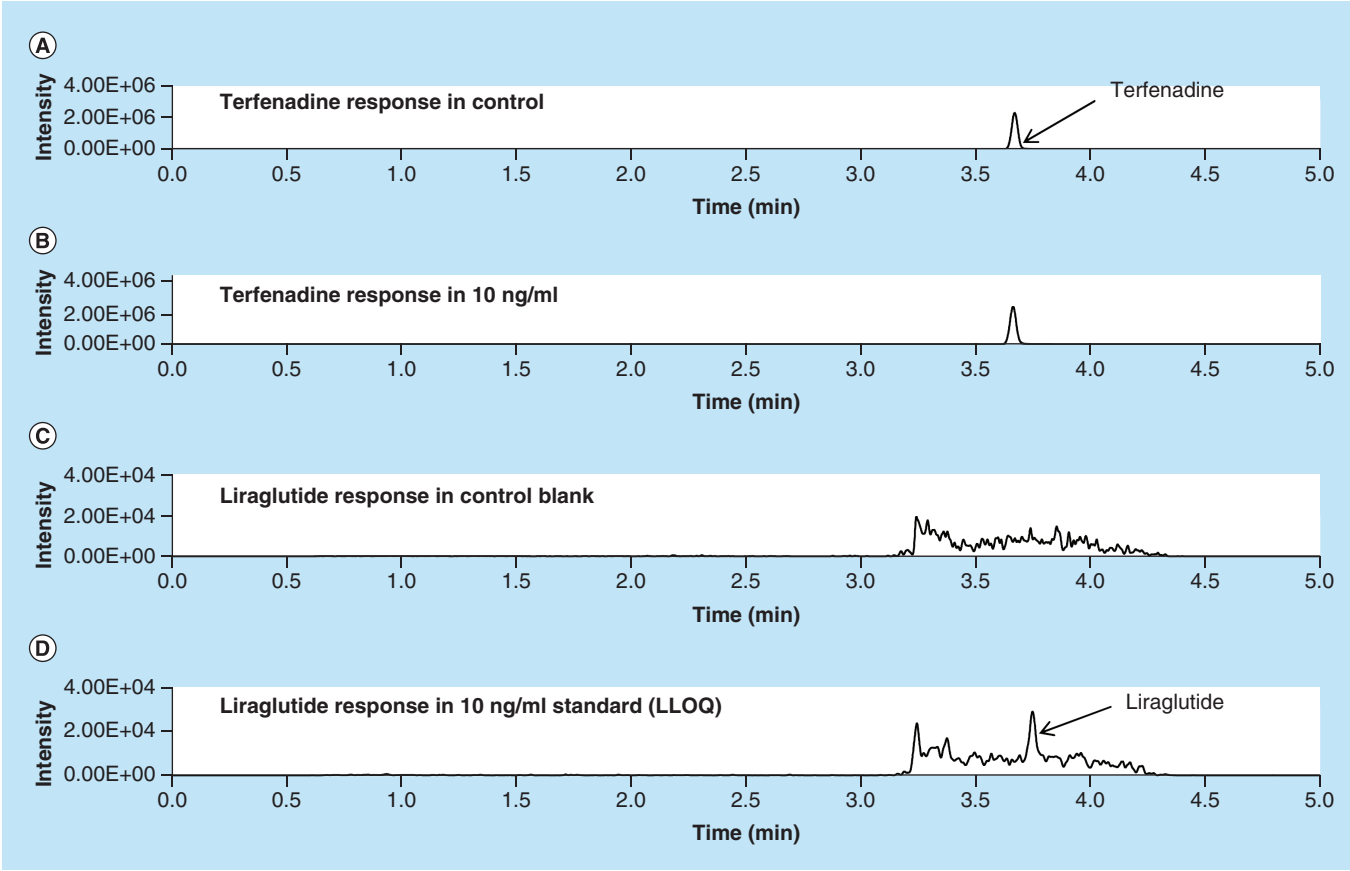

Figure 2. Assessment of assay selectivity in blank matrix. Chromatograms of the multiple reaction monitoring channels of internal standard terfenadine **(A & B)** and analyte liraglutide **(C & D)** in an extracted plasma blank **(A & C)** and a 10 ng/ml standard **(B & D)**.

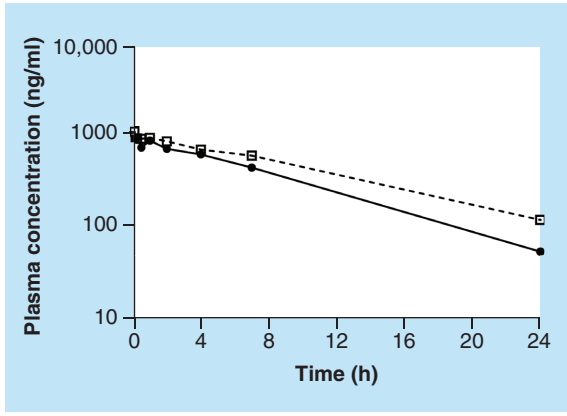

Figure 3. Plasma concentration time profiles of liraglutide following intravenous administration at 30 μg/kg to male cynomolgus monkeys (n = 2). The concentrations for each animal are plotted individually.

Discussion

As the LC eluent flow through an ESI source is reduced, analyte desolvation and ionization become more efficient [30]. Furthermore as the flow is reduced the electrospray emitter can be aligned closer to the instrument entrance resulting in the more efficient transmission of the ion current into the mass spectrometer [31]. The result is improved sensitivity which lowers the analyte's LOD particularly as flow rates are reduced below 10 μl/min provided that the chromatographic separation remains efficient at the low flow rate and that chromatographic dead volumes are managed. It is the time and expertise required to optimize these systems combined with the accelerated pace and number of compounds supported in the discovery phase of pharmaceutical development that has prevented routine

use of very low flow electrospray systems in discovery support laboratories. In the work presented here, we utilized the Water's tile-based IonKey/MS system where the chromatographic separation occurs in a 50 mm × 150 µm channel that has been etched into a ceramic tile and filled with 1.8 µm o.d. chromatographic particles. The channel terminates at an electrospray emitter and is encased in a 'key' which also provides the necessary ports for fluidic, gas and electrical connections. The key is inserted and engaged with the ionization source housing to make low dead volume fluidic connections and align the electrospray emitter with the instrument entrance orifice [26]. The ease, quality and reproducibility of the alignment and fluidic connections combined with the diameter of the separation channel enable the generation of high-resolution chromatographic separations in the 1–10 µl/min flow range.

Inclusion of a 16-carbon fatty acid moiety in liraglutide increases binding to plasma proteins, resulting in a long terminal $t_{1/2}$, which permits administration of a low total daily dose (1.8 mg) to humans. The human plasma protein binding of liraglutide is >98.9% at its efficacious clinical concentrations (~10–34 nM), with specific binding to human serum albumin and α1-acid glycoprotein estimated to be 99.4 and 99.3%, respectively [18]. Consequently, selection of an optimal solvent for protein precipitation and liraglutide extraction from plasma was critical for the development of the bioanalytical methodology. During initial method development, extraction efficiencies of three solvents – methanol, ethanol and acetonitrile were evaluated to determine which would provide the best recovery of liraglutide from cynomolgus monkey plasma. The recovery of liraglutide from cynomolgus monkey plasma following protein precipitation with acetonitrile and centrifugation was very low, approximately 28%, which significantly impacted the lowest level of quantitation that could be achieved. Recovery was significantly improved when methanol and ethanol were used as the precipitating agents. Consequently, ethanol was selected as the extraction solvent for this application.

Addition of acetonitrile to plasma samples is a common technique to effectively precipitate most of the proteins from plasma and thereby reduce sample matrix interferences [32]. This approach has the additional advantage of releasing small-molecule, protein-bound analytes which are then extracted by the organic supernatant. The precise reason(s) for the low extraction efficiency with acetonitrile remains unclear in the present work. It is noteworthy to point out that the pellets obtained upon plasma protein precipitation in samples treated with acetonitrile were very dense compared with the pellets obtained upon treatments of the plasma samples with methanol or ethanol, respectively. It is possible that the small surface area of the acetonitrile pellet may have limited the release of liraglutide, while the larger surface area of the ethanol and methanol pellets allowed for a more complete release of the bound liraglutide, resulting in higher recovery.

Ideally, radio or stable isotope-labeled ISs are used in LC–MS/MS bioanalysis so that matrix interferences and ionization efficiencies can be completely accounted for. Our LC–MS/MS assay was primarily developed to support fit-for-purpose discovery studies and stable-labeled liraglutide of sufficient purity for use as an IS is not commercially available. Therefore, we chose the small molecule terfenadine for an IS based on its similar retention time under the chromatographic conditions and its availability in our laboratory. Polson *et al.* reported that a 2:1 ethanol:human plasma extraction results in removal of 88.1% of plasma proteins, leaving the remaining proteins to potentially cause matrix effects [32]. Thus, it was necessary to understand what, if any matrix effects were present and determine how well our selected IS (terfenadine) was accounting for these in our samples. Analysis of the analyte peak areas of blank individual plasma lots extracted at a 2:1 ratio of ethanol-to-plasma and postextraction spiked with liraglutide revealed that there was indeed a large matrix effect, with approximately 80% of the analyte signal being suppressed. This observation was consistent for all individual plasma lots evaluated. For the same samples, analysis of the peak area ratios of liraglutide/terfenadine (analyte/IS) showed a lowering of the change in area ratio response across all lots, indicating the IS does help reduce the matrix effects, but does not completely normalize the matrix effect-induced variability of the peak area ratios. A comparison of the mean lot-to-lot percent lowering demonstrates a range of -32.5 to -9.55% with a median value of 22.1 indicating there remains some subject-to-subject variability even with the IS (Table 1). Therefore standard and QC samples were prepared in pooled monkey plasma in an effort to further reduce the impact of the remaining response variability. Considering that this assay was being developed to support discovery pharmacology and pharmacokinetics studies in animals, this variability was deemed acceptable. Further compensation for ionization suppression may be possible if the IS is changed to an analog peptide which may be more easily synthesized with high purity than a stable-labeled liraglutide [33].

Concentrations for calibration standards (10–5000 ng/ml [2.6–1330 nM]) were selected to cover plasma liraglutide concentrations in monkey pharmacokinetics studies that would reflect the efficacious steady state liraglutide concentration (128 ng/ml, 34 nM) in humans over 24 h [34]. Assay validation consisted of inter- and

intraday accuracy and precision and selectivity. The accuracy relative to nominal concentration of all back calculated, ($1/x^2$ weighted regression), standard concentrations in the validation batch runs met the generally accepted criteria of ±15% except for one standard in the third batch run (-17.8%) and the linear regression coefficients (R^2) were ≥0.98.

In its current format, the bioanalytical assay was successfully utilized to characterize liraglutide pharmacokinetics following iv. bolus administration of a 30 μg/kg dose to male cynomolgus monkeys since these parameters are not available in the literature. The iv. pharmacokinetics of liraglutide in monkey comprise of very low total CL_p (0.0447 ml/min/kg) and V_{dss} (21.6 ml/kg) in monkeys, and a relatively long terminal elimination $t_{1/2}$ of 6.59 h, a trend that is similar to the previously published pharmacokinetics parameters (CL_p = 0.1 ml/min/kg, V_{dss} = 86 ml/kg, $t_{1/2}$ = 9.1 h) obtained upon iv. administration of a 5 μg/kg dose of liraglutide to human subjects [35]. As such, the corresponding plasma AUC following iv. administration of 30 μg/kg liraglutide to monkeys was 11,500 ng.hr/ml, which corresponded to an average total concentration of approximately 128 nM (AUC × 1000/24/liraglutide MW), a 3.7-fold higher value than the one (34 nM) achieved in humans at the efficacious dose (1.8 mg) used to treat T2DM.

Conclusion

In summary, we have disclosed a validated LC–MS/MS-based assay for the detection and quantitation of the peptide-based GLP-1 receptor agonist of the GLP-1 liraglutide in cynomolgus monkey plasma, which allowed a detailed characterization of liraglutide pharmacokinetics in monkeys after iv. dosing.

Future perspective

By taking advantage of recent advances in microflow technology, our LC–MS/MS assay could detect plasma liraglutide concentrations as low as 10 ng/ml (2.6 nM) with a minimal volume (~50 μl) of plasma, which was prepared by simple protein precipitation with an organic solvent. While the published immuno-based assay has a lower LOD, the LC–MS/MS assay, in its current format, was adequate for the characterization of the monkey pharmacokinetics of liraglutide following iv. administration. Furthermore, using the LC–MS/MS assay, we were able to successfully reproduce the average steady state liraglutide plasma concentrations achieved in humans over 24 h (at the T2DM efficacious dose of 1.8 mg) in monkeys. This assay proved to be a simple, cost- and time-effective

Summary points

Background

- Liraglutide (Victoza®, Saxenda®) belongs to a class of incretin- and peptide-based glucagon-like peptide-1 receptor agonists for the treatment of Type 2 diabetes mellitus and obesity.
- A cost-effective and sensitive LC–MS/MS method was developed and validated for the quantitation of liraglutide in plasma samples.

Experimental

- The LC–MS/MS assay utilized Water's tile-based IonKey/MS system that integrates sub-10 μl/min flow rate chromatographic separations with ESI.
- Samples are prepared by simple protein precipitation with ethanol.
- The small molecule terfenadine is used as the internal standard as a low cost and available alternative to a stable-labeled or structural analog.
- The assay was validated according to US FDA guidelines.
- The pharmacokinetic parameters after an intravenous bolus of liraglutide in monkey are determined.

Results & discussion

- The increased ionization and ion transmission efficiencies enabled by 3-μl/min flow to the electrospray source enables the simplification of the sample preparation procedure.
- The assay met FDA validation criteria.
- The internal standard terfenadine moderates but does not completely compensate for matrix-induced ionization variability but is adequate for studies that support discovery.
- The bioanalytical assay was used to successfully characterize liraglutide pharmacokinetics following intravenous bolus administration to male cynomolgus monkeys; values that have not been published in the literature.

Conclusion

- The described method utilizes the Waters IonKey system and was successfully validated and used to support a discovery monkey pharmacokinetic study.

approach for liraglutide analysis in monkey plasma, which could easily be adapted for other preclinical species and human. It is noteworthy to point out that during the write up of this work, we came across recent work from Meng *et al.* [36] that disclosed an LC differential mobility spectrometry tandem MS with multiple ion-monitoring detection method for the quantitation of liraglutide in dog plasma. In contrast to our work, the sample preparation involved anion-exchange SPE, which somewhat improved the LLOQ to 1 ng/ml.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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