Original article

Ultra-sensitive bioanalysis of the therapeutic peptide exenatide for accurate pharmacokinetic analyses at effective plasma concentrations utilizing UPLC-MS/MS

Max Sauter, Philipp Uhl, Jürgen Burhenne[*], Walter E. Haefeli

Department of Clinical Pharmacology and Pharmacoepidemiology, Heidelberg University Hospital, Im Neuenheimer Feld 410, 69120, Heidelberg, Germany

A B S T R A C T

Exenatide is the first approved glucagon-like peptide 1 receptor agonist subcutaneously or intramuscularly injected for the treatment of type 2 diabetes mellitus. Typical therapeutic plasma concentrations are in the low pg/mL range, therefore requiring ultra-sensitive quantification. To enable the accurate evaluation of pharmacokinetic studies, we established a UPLC-MS/MS assay with a lower limit of quantification (LLOQ) of 5 pg/mL (1.2 pM) using 200 μL of plasma, validated according to FDA's and EMA's pertinent guidelines. Exenatide was isolated from plasma with solid phase extraction utilizing anion-exchange sorbent. Quantification was performed with positive electrospray ionization tandem mass spectrometry in the selected reaction monitoring mode. The calibrated concentration range of 5–10,000 pg/mL was linear showing correlation coefficients >0.99. Interday and intraday accuracy ranged from 97.5% to 105.4% with corresponding precision of <10.9%. Accuracy at the LLOQ ranged from 93.0% to 102.5% with corresponding precision of <15.9%. Because of the validity of a 10-fold dilution QC (accuracy 111.2%), the assay is suitable for exenatide quantification up to 100,000 pg/mL. The ultra-sensitive assay's applicability was demonstrated by the quantification of exenatide plasma concentrations and pharmacokinetics after intravenous and nasal administration to beagle dogs.

© 2020 Xi'an Jiaotong University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Glucagon-like peptide 1 (GLP-1) receptor agonists are recommended early in the management of type 2 diabetes mellitus (T2DM) [1]. Exenatide (synthetic exendin-4), a 39-amino acid peptide with an amide C-terminus (sequence: H G E G T F T S D L S K Q M E E E A V R L F I E W L K N G G P S S G A P P P S; $C_{184}H_{282}N_{50}O_{60}S$, 4,186.6 g/mol), originates from the saliva of the Gila monster [2,3] and is the first approved GLP-1 receptor agonist. In patients with T2DM, glycemic control is improved by exenatide, which reduces fasting and postprandial glucose-concentrations through glucose-dependent insulinotropic and glucagonostatic effects and increase of β-cell mass, while further fostering weight loss via reduction of energy intake by inducing satiety and delaying gastric emptying [2,4–6].

Exenatide is available as immediate release subcutaneous injection for twice daily administration (Byetta®) [7] and as extended release weekly subcutaneous injection formulated in microspheres (Bydureon®) [8] which frequently cause injection site reactions [9–14]. Exenatide exerts efficacy in the low picomolar range showing a half maximal effective concentration (EC_{50}) on fasting plasma glucose of 56.8 pg/mL (~14 pM) [15], and typical peak (for the therapeutic 10 μg dose) and steady-state plasma concentrations around 200 to 300 pg/mL (~50 to 70 pM) [2,15–19]. With the latest approval of the first oral GLP-1 receptor agonist semaglutide (Rybelsus®) [20,21], investigations aiming at the development of alternative routes of exenatide administration to avoid the frequently associated injection site reactions are anticipated to receive increased attention.

Because of today's availability of sufficiently sensitive tandem mass spectrometers, quantification of peptides is increasingly performed by MS/MS methodologies due to their advantages of a wider dynamic range, often great accuracy, and especially superior specificity due to the lack of cross-reactivity in comparison to the traditionally performed immunoassays. However, the sensitive bioanalysis of large peptides using MS/MS is challenging because of their high molecular weight, multiple charge distribution, and

Peer review under responsibility of Xi'an Jiaotong University.

* Corresponding author.
  E-mail address: juergen.burhenne@med.uni-heidelberg.de (J. Burhenne).

typical lack of intense, specific fragments in collision-induced dissociation (CID) sourcing from the multitude of possible bond breakages.

Thus far, only few assays for the bioanalysis of exenatide have been published that rely on LC-MS methodologies and none is suitable for accurate pharmacokinetic analyses in the therapeutic range [22–24]. Such assays require lower limits of quantification (LLOQ) that are at least tenfold lower than observed peak plasma concentrations. The lowest reported LLOQ of LC-MS-based assays is 50 pg/mL (24 pM) for an LC-HRMS assay avoiding CID [23], while the lowest LLOQ for immunologic exenatide quantification is 10 pg/mL [5,18].

To enable reliable pharmacokinetic investigations of exenatide and allow formulation development for alternative routes of administration, we established an ultra-sensitive UPLC-MS/MS assay for plasma exenatide quantification with a remarkably low LLOQ of 5 pg/mL (1.2 pM). The assay was validated according to the pertinent recommendations of the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) [25,26]. Its suitability for pharmacokinetic studies and formulation development was demonstrated by the quantification of plasma exenatide after nasal and intravenous administration to beagle dogs enabling the determination of exenatide's absolute nasal bioavailability (F).

2. Materials and methods

2.1. Beagle dog study and plasma sample generation

The study was carried out at Citoxlab France (Evreux, France) in accordance with national and European guidelines for the care and use of laboratory animals (European regulations 2010/63/EU and the French decret No. 2013-118 on the protection of animals used for scientific purposes). The project (#4786; animal facility D-2741001) has been approved by the French Ministry of Higher Education and Research (Ministère de l'Enseignement Supérieur et de la Recherche) under the ethical committee number C2EA-48. Four male beagle dogs received an intravenous bolus injection of 1 mL of a 0.1 mg/mL solution of exenatide (100 μg) in phosphate buffered saline followed after a wash-out of one week by one nasal spray puff of 100 μL of a 0.6 mg/mL exenatide solution (60 μg) in phosphate buffered saline. Blood samples were drawn into heparinized tubes (2 mL) before administration and 0.25, 0.5, 1, 2, 4, 6, 12, and 24 h after administration, immediately centrifuged at 2,500 ×g for 10 min while refrigerated to 4 °C, and the plasma was stored at -80 °C until analysis.

2.2. Drugs, chemicals, solvents, and materials

Exenatide acetate (92.6%) was obtained from Bachem AG (Bubendorf, Switzerland). Isotopically labeled internal standard (IS) [$^{13}C_6$,^{15}N]-Leu(10,21,26)-exenatide, which has all leucines in the sequence isotopically labeled (resulting mass difference 21 Da), was purchased from Peptide Specialty Laboratories GmbH (Heidelberg, Germany). Remaining reagents and solvents (water, methanol (MeOH), acetonitrile (ACN), and formic acid (FA)) were purchased from Biosolve (Valkenswaard, The Netherlands) in the highest available purity. Blank beagle plasma, supplied by Innovative Research (Novi, MI, USA), was obtained from Dunn Labortechnik GmbH (Asbach, Germany).

2.3. Standard solutions

For preparing calibration and QC stock solutions, exenatide was independently weighed into plastic (polypropylene) reaction tubes to circumvent adsorption observed for glass vessels, and

subsequently dissolved in 4 mL ACN/water (1/1, v/v) + 0.1% FA. Solutions were then diluted 100-fold with ACN/water (1/1, v/v) + 0.1% FA. Calibration spike-solutions were prepared from the diluted stock at concentrations of 40, 120, 480, 1,600, 6,400, 20,000, and 80,000 pg/mL in ACN/water (1/1, v/v) + 0.1% FA (corresponding to sample concentrations of 5, 15, 60, 200, 800, 2,500, and 10,000 pg/mL). QC spike solutions were prepared accordingly at 120, 4,800, 60,000, and 160,000 pg/mL (corresponding to sample concentrations of 15, 600, 7,500, and 20,000 pg/mL for QC A, B, C, and D, respectively). The IS spike solution was prepared at 25,000 pg/mL (corresponding to a sample concentration of 3,125 pg/mL). Solutions were kept at 4 °C and were stable for at least 2 months.

2.4. Plasma sample preparation

To 200 μL of plasma in 2 mL reaction tubes, 25 μL of IS spike solution and 25 μL of the respective calibration or QC spike solution was added for the preparation of calibration and QC samples. Study plasma samples (200 μL) were spiked with 25 μL of IS and 25 μL of ACN/water (1/1, v/v) + 0.1% FA for volume compensation. To enable the determination of plasma concentrations above the calibrated range, which are anticipated to occur early after intravenous injection, a dilution QC was validated. Dilution QC D samples were generated by preparing QC D samples without addition of IS by addition of 25 μL of QC D spike solution to 175 μL of plasma and the subsequent dilution of 20 μL of these samples with 180 μL of blank plasma, which were then spiked similar to study samples. For extraction, samples were treated with 75 μL of 25% aqueous ammonia, transferred to wells of an Oasis® MAX μElution Plate (anion exchange; Waters, Milford, MA, USA), and loaded onto the sorbent by applying positive pressure (positive pressure unit; Waters, Milford, MA, USA). Wells were washed with 100 μL of ACN/water (1/1, v/v) containing 5% ammonia followed by 100 μL of ACN/water (1/4, v/v) + 0.05% FA. Subsequently, exenatide was eluted from the solid phase into wells of a 96-well collection plate (800 μL; Waters, Milford, MA, USA) with two times 30 μL of ACN/water/MeOH (2/1/1, v/v/v) containing 5% FA. To the wells of the collection plate, 40 μL of water was added, the plate was sealed, shaken, and samples were injected onto the UPLC-MS/MS system.

2.5. Instrumental analysis parameters

A UPLC-MS/MS system (Waters, Milford, MA, USA) consisting of a triple stage quadrupole mass spectrometer (Waters Xevo TQ-XS with Z-spray source) equipped with an Acquity classic UPLC® system (Waters, Milford, MA, USA) was used for mass spectrometric detection. Chromatographic separation was performed on a Waters Acquity BEH C_{18} peptide column (300 Å, 2.1 mm × 50 mm, 1.7 μm) heated to 80 °C using a flow rate of 0.5 mL/min and an injection volume of 20 μL. The eluent consisted of 0.1% (v/v) aqueous FA with 5% ACN (aqueous eluent; A) and ACN including 0.1% FA (ACN eluent; B). Initial conditions of 80% A/20% B were kept for 0.1 min followed by a change to 67% A/33% B within 1.9 min. Subsequently, the ratio was changed to 5% A/95% B within 0.5 min, adjusted to 50% A/50% B in 0.5 min, changed back to 5% A/95% B within 0.5 min and kept for an additional 0.5 min before returning to starting conditions in 0.5 min. The initial conditions were kept for equilibration while the subsequent injection was prepared (1 min), which resulted in a total cycle time of 5.5 min.

The Z-spray ionization parameters were manually optimized and the Xevo TQ-XS was tuned to exenatide and the IS using the MassLynx V4.2 system software (Waters, Milford, MA, USA) with integrated IntelliStart procedures. Selected reaction monitoring (SRM) measurements were performed utilizing Ar for CID in

Table 1
Optimized parameters for the detection of exenatide using UPLC-MS/MS with positive heated ESI and SRM.

Parameter	Exenatide	[$^{13}C_6$,^{15}N]-Leu(10,21,26)-exenatide
Spray voltage (V)	700	700
Cone voltage (V)	20	20
Source temperature (°C)	150	150
Desolvation gas flow (N_2) (L/h)	1,000	1,000
Desolvation temperature (°C)	600	600
SRM transition (m/z)	838.3 → 948.7	842.5 → 954.0
Dwell time (ms)	100	100
Collision energy (V)	18	18
Collision gas flow (Ar) (mL/min)	0.15	0.15

ESI: Electrospray ionization; SRM: selected reaction monitoring.

positive ion mode. Mass spectrometric characteristics are shown in Table 1.

2.6. Validation of the analytical methods

The assay was validated following the pertinent recommendations published by the FDA and EMA [25,26]. Accuracy (expressed in percent) was calculated as the ratio of mean concentrations measured in individual batches divided by the nominal value. Precision (expressed in percent) was determined from the ratio of standard deviation (SD) and mean measured concentration. Validity of the assay was demonstrated by analysis of three validation batches with each batch containing at least eight calibration samples and at least 24 QC samples at different concentrations (LLOQ, QC A, B, and C, as well as additional dilution QC D; six-fold each). Blank plasma samples from six different beagle dogs, which were processed without addition of analyte and IS, were used for testing the assay's selectivity by evaluating the baseline at the retention time of the analyte.

Extraction recovery rates from plasma were assessed from QC samples A to C in three-fold determinations by the ratio of their respective peak areas and the respective peak areas from blank plasma spiked after extraction (representing 100% analyte amount in identical matrix). Matrix effects were determined also in three-fold determination for QC A to C by comparing peak areas of blank plasma samples spiked after extraction with the respective peak areas of matrix-free solvent spiked with the identical amount [27].

Short-term and long-term stability of exenatide in plasma at room temperature and -40 °C has already been demonstrated [22]. However, during validation, the stability of exenatide in the pg/mL range was assessed for plasma samples stored at -25 °C for 3 weeks, which well covers the storage time of the beagle dog study plasma samples, as well as in three freeze-and-thaw cycles using QC samples A to C. Stability of the extracts in the autosampler was evaluated by repeated analysis of QC A to C after remaining in the autosampler at 10 °C for 24 h.

To evaluate the assay's applicability to human studies, specificity, recovery, and matrix effect were also determined in human plasma (citrate).

2.7. Calculations and statistical methods

Calibration curves were calculated with weighted linear regressions ($1/x^2$) from the peak area ratios of the analyte and IS of calibration samples using Waters TargetLynx V4.2 software (Waters, Milford, MA, USA). Non-compartmental pharmacokinetic parameters were determined utilizing Thermo Kinetica Version 5.0 (Thermo Fisher Scientific, Waltham, MA, USA); maximum plasma concentration (C_{max}) and time to C_{max} (t_{max}) was directly obtained from the raw data, terminal elimination half-life ($t_{1/2}$), AUC extrapolated to infinity, apparent volume of distribution at steady state (V_{ss}/F), and apparent oral clearance (Cl/F) were calculated by a mixed log-linear model. Absolute exenatide bioavailability was calculated as AUC(nasal) ÷ AUC(intravenous) × Dose(intravenous) ÷ Dose(nasal) × 100%. Standard calculations were performed with Microsoft Office Excel 2010 (Mountain View, CA, USA).

3. Results and discussion

3.1. Mass spectrometric and chromatographic characteristics

Positive electrospray ionization of exenatide (4,186.6 Da) yielded the [M+5H]$^{5+}$ signal at m/z 838.3 as most intense ion (m/z 842.5 for the IS). In contrast to most peptides that usually show immonium ions of single amino acids as most abundant product ions (especially at high collision energy) as well as a multitude of larger fragments in CID, exenatide shows two large fragments (apart from water loss observed at m/z 834.7) as explicitly most intense CID product ions that both correspond to the identical dissociation-reaction (Fig. 1). These product ions were observed at m/z 948.7 and at m/z 396.2, respectively, and constituted the b_{35} fragment ($z = 4$) and the y_4 fragment ($z = 1$), respectively. Their identity and charge were confirmed by high-resolution mass spectrometric analysis (deviation to calculated m/z value < 20 ppm) on a Waters Xevo G2-XS QTof mass spectrometer (Waters, Milford, MA, USA). Fig. 1 depicts the product spectra of the [M+5H]$^{5+}$ signal of exenatide (m/z 838.3) and the dissociation reaction corresponding to the selected quantifier transition m/z 838.3 → m/z 948.7. For the isotopically labeled IS, the corresponding mass transition was monitored at m/z 842.5 → m/z 954.0. The mass shift of 5.3 Da of the CID fragment of the IS was in line with the $z = 4$ signal of the b_{35}-fragment comprising all three [$^{13}C_6$,^{15}N]-leucines (mass difference 21 Da; Fig. 1).

The superior intensity of the two abundant product ions (b_{35} and y_4 fragment) indicates that one peptide bond in exenatide is substantially more labile and predestined for dissociation in CID. As a consequence of the favored single dissociation-reaction, exenatide can be quantified ultra-sensitively by MS/MS despite its high molecular weight and numerous peptide bonds.

Chromatographic separation of exenatide was performed on a Waters BEH peptide C_{18} UPLC® column with a pore width of 300 Å which together with heating to 80 °C facilitated interaction of the large peptide exenatide with the solid phase to achieve sharp chromatographic peaks. A gradient from 80% to 67% aqueous eluent and a parallel increase of the ACN eluent proportion from 20% to 33% within 2 min resulted in a fast separation gradient and a peak width at baseline of 5 s. Dwell times of 100 ms for analyte and IS transition yielded approximately 25 data points per peak and, hence, well resolved single mass traces (Fig. 2). Because of initially observed carry-over affecting successive analyses, a second gradient for cleaning purposes was implemented into the
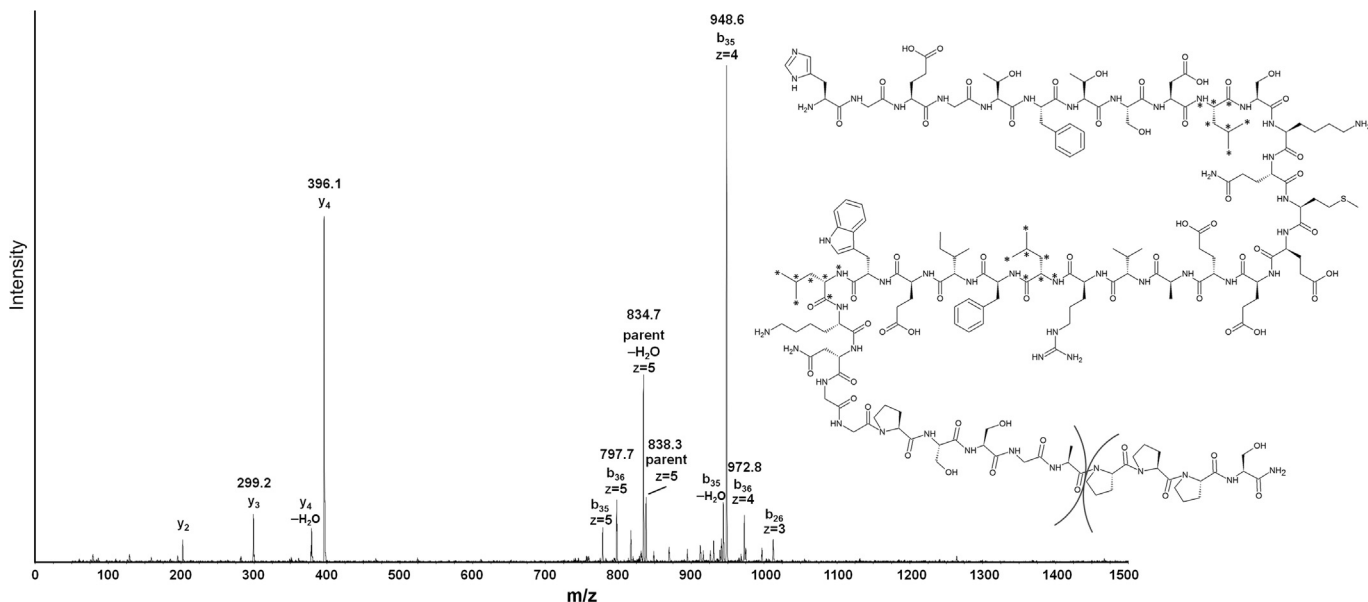
Fig. 1. Positive product spectrum of exenatide using collision-induced decomposition at 18 V. The grey brackets in the exenatide structure depict the preferred dissociation reaction corresponding to the m/z 838.3 → m/z 948.7 (b_{35}) and m/z 838.3 → m/z 396.2 (y_4) transition. The positions of the isotopic labels in the corresponding internal standard are marked with an asterisk.

chromatographic method, which reduced the carry-over to levels well below the required 20% peak area of the LLOQ signal.

3.2. Extraction by protein precipitation

Exenatide is an acidic peptide bearing more carboxyl functions than amino groups, rendering it accessible to anion exchange chromatography, similar to the GLP-1 receptor agonist liraglutide

[28]. Therefore, isolation of exenatide from plasma was performed by solid phase extraction (SPE) using mixed mode strong anion exchange and reverse phase sorbent, which yielded quantitative recovery (99.9% to 110.8% for QC A to C). SPE anion exchange isolation of exenatide allowed rigorous washing and resulted in little remaining matrix interference, which could be separated by optimized chromatographic conditions. The matrix effect for QC A to C was negligible with determined values between −7.1% and
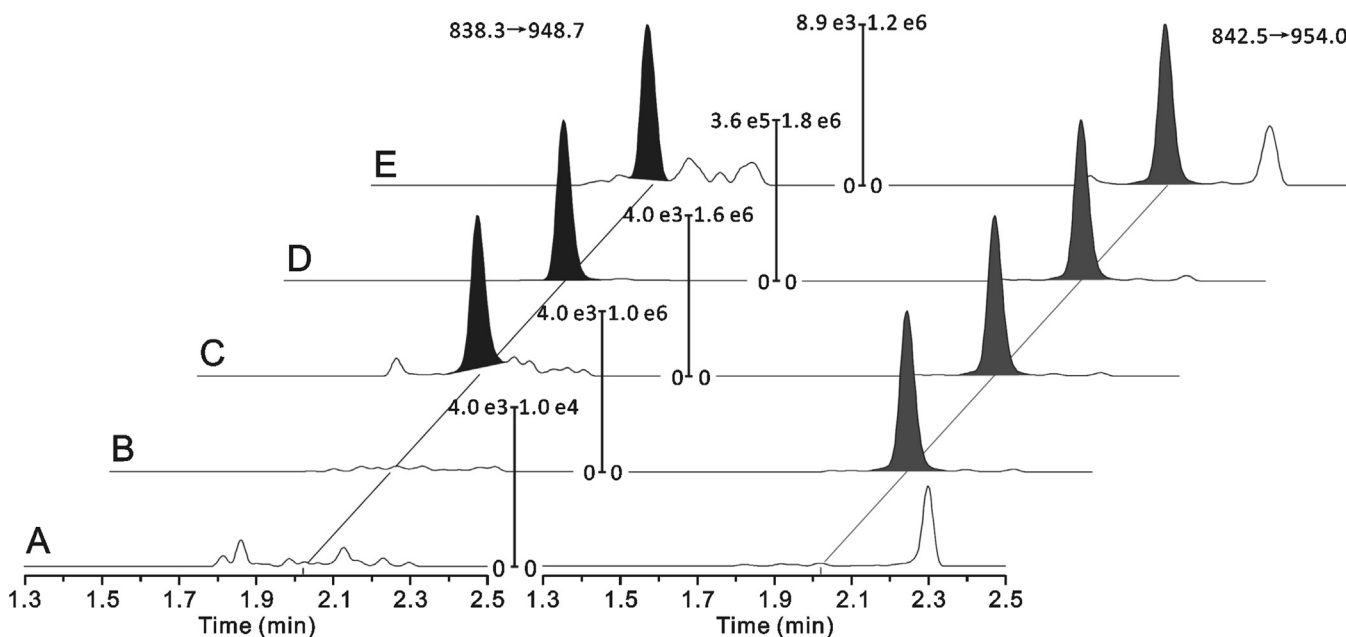

Fig. 2. Selected UPLC-MS/MS chromatograms of processed plasma samples with exenatide quantifier transition on the left (black filling) and IS transition on the right (grey filling). Intensities were normalized to the highest signal except for the exenatide quantifier transition of blank and IS spiked plasma which were normalized to the intensity of the lower limit of quantification (LLOQ) signal. (A) blank plasma sample, (B) plasma sample with added IS, (C) plasma sample at LLOQ concentration (representing 5 pg/mL), (D) plasma sample at QC B concentration (representing 600 pg/mL), and (E) plasma sample of beagle dog #1 15 min after nasal administration of 60 μg exenatide (quantified exenatide concentration 15.8 pg/mL).

Table 2
Summary of quality control results for exenatide in plasma.

Batch	Parameters	LLOQ (5.00 pg/mL)	QC A (15.0 pg/mL)	QC B (600 pg/mL)	QC C (7,500 pg/mL)
Within-batch					
1	Mean (pg/mL)	4.65	15.0	603	7,680
	Accuracy (%)	93.0	100.0	100.5	102.4
	Precision (%CV)	15.2	3.37	4.21	2.83
2	Mean (pg/mL)	4.85	14.9	614	7,905
	Accuracy (%)	97.0	99.4	102.3	105.4
	Precision (%CV)	9.07	8.32	2.66	2.68
3	Mean (pg/mL)	5.13	14.6	606	7,693
	Accuracy (%)	102.5	97.5	100.9	102.6
	Precision (%CV)	15.9	10.9	7.06	8.66
Batch-to-batch					
	Mean (pg/mL)	4.86	14.8	607	7,763
	Accuracy (%)	97.2	98.9	101.2	103.5
	Precision (%CV)	13.4	7.64	4.83	5.01

CV: Coefficient of variation; LLOQ: Lower limit of quantification; QC: Quality control.
$n = 5$ replicates at LLOQ and each QC concentration.

Table 3
Results of the incurred sample reanalysis of 9 plasma samples after intravenous administration.

Sample	Original analysis (pg/mL)	Incurred reanalysis (pg/mL)	Deviation (%)
Dog1 15 min	31,984	26,739	−16.4
Dog1 30 min	18,693	17,741	−5.1
Dog2 15 min	38,013	37,203	−2.1
Dog2 30 min	22,063	22,774	3.2
Dog3 15 min	33,936	39,227	15.6
Dog3 30 min	26,266	23,743	−9.6
Dog3 60 min	13,172	12,829	−2.6
Dog4 15 min	40,817	39,019	−4.4
Dog4 30 min	22,412	21,165	−5.6

3.6%.

Anion-exchange purification of peptides conveys the significant advantage of yielding acidic extracts, which is beneficial for peptide stability. Therefore, neutralization and excessive dilution steps can be avoided which, combined with the utilization of the μ-elution format, resulted in concentrated sample extracts suitable for ultra-sensitive quantification.

3.3. Validation results

The anion exchange extraction combined with UPLC-MS/MS quantification of exenatide was in complete compliance with the pertinent guidelines of FDA and EMA [25,26]. Selectivity was demonstrated in blank matrices from six individual beagle dogs by absence of signals at the analytes' retention times. Fig. 2 shows typical chromatograms of blank plasma as well as plasma samples spiked with IS, at LLOQ concentration, at QC B concentration, and one beagle dog plasma sample 15 min after nasal administration of 60 μg exenatide. The correlation coefficients (r^2) of all calibration curves were >0.99. Intraday accuracies (QC A to C) varied between 97.5% and 105.4% with corresponding precisions <10.9% while interday accuracies ranged from 98.9% to 103.5% with corresponding precisions <7.7%. The accuracy at the LLOQ varied between 93.0% and 102.5% intraday and was 97.2% interday. The corresponding precisions were <15.9% and 13.4%, respectively. A summary of the quality control results during validation is given in Table 2.

For quantification of higher exenatide plasma concentrations occurring early after intravenous administration, a 10-fold dilution with blank plasma (QC D) was validated using 20 μL of plasma.
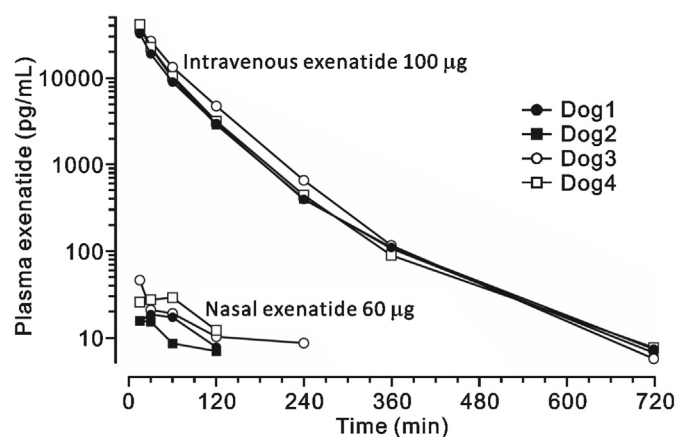
Fig. 3. Exenatide plasma concentration-time profiles in beagle dogs after intravenous (100 μg) and nasal (60 μg) administration.

Accuracy of the dilution QC D was 111.2% with corresponding precision of 1.09%, proving the assay's applicability to quantify plasma exenatide up to 100,000 pg/mL (100 ng/mL). The reliability of exenatide quantification was demonstrated by an incurred sample reanalysis of 9 study samples originating from all four exposed beagle dogs which all showed deviations of <16.5% (2.1%–16.4%) compared to the original analysis, being well within the requirements of the FDA and EMA (Table 3).

Due to the utilization of an isotopically labeled IS, which accurately accounts for recovery and matrix effects, the assay is in principle applicable to human plasma determinations. Therefore,

Table 4
Single dose pharmacokinetics of exenatide in beagle dogs after intravenous bolus injection and after nasal administration.

Exenatide dose and route of administration	Weight of dog (kg)	C_{max} (pg/mL)	AUC (pg/mL · h)	V_Z (L/kg)	Cl (mL/min)	$t_{1/2}$ (min)	F (%)
Intravenous	1 (10.1)	31,984	25,394	0.62	65.6	66.5	100
100 µg	2 (10.2)	38,013	28,483	0.54	58.5	65.1	100
	3 (9.5)	33,936	34,397	0.44	48.5	59.9	100
	4 (9.1)	40,817	30,024	0.58	55.5	65.3	100
Nasal	1 (10.1)	18.7	37.4	0.48	64.1	52.7	0.24
60 µg	2 (10.2)	15.9	27.3	0.30	58.6	36.3	0.16
	3 (9.5)	46.6	74.3	0.49	48.5	67.2	0.36
	4 (9.1)	29.3	57.9	0.42	55.3	47.8	0.32

AUC: area under the concentration-time curve; Cl: clearance corrected for F; C_{max}: maximal plasma concentration; F: absolute bioavailability calculated as AUC(oral) ÷ AUC(iv) × 100 % × dose(iv) ÷ dose(oral); $t_{1/2}$: half-life; V_Z: volume of distribution corrected for F.

the possible LLOQ of our assay in human plasma and the required plasma volume are primarily dependent on the extraction characteristics and matrix interferences. Because the extraction characteristics of exenatide in human plasma (recovery of 84.0% to 87.8% and a matrix effect of −9.3% to −5.8%) are similar to beagle plasma and due to the absence of interfering signals in blank plasma from six different individuals, applicability of the presented assay is indicated also for plasma exenatide quantification in human studies.

3.4. Stability

Stability of plasma exenatide was demonstrated by accurate quantification of freeze-and-thaw samples of QC A to C, which showed accuracies between 93.8% and 101.7%. Additionally, QC samples A to C stored at −25 °C for 3 weeks showed accuracies ranging from 105.4% to 112.0%, confirming plasma exenatide stability for storage under these conditions. Stability in plasma has already been demonstrated for 1 month at −40 °C and 2 h at room temperature [22].

Plasma extracts were stable after remaining in the Sampler Manager for 24 h, which is sufficient for the course of analysis also for large batches and favorable for high throughput quantification of pharmacokinetic samples. The stability of exenatide in the stock and standard solutions was confirmed by the quantification of freshly prepared QC A to C samples (from an independent new weighing) with calibration solutions stored at 4 °C for 2 months, which revealed accuracies of 106.0% to 111.7%.

3.5. Pharmacokinetics of exenatide after intravenous and nasal administration to beagle dogs

Fig. 3 shows the exenatide plasma concentration-time profiles after intravenous and nasal administration to four beagle dogs (bodyweight 9.1 to 10.2 kg) and the corresponding non-compartmental pharmacokinetic analyses are given in Table 4. After intravenous bolus injection of 100 µg, exenatide's mean volume of distribution was 0.54 L/kg (range: 0.44–0.62) and mean clearance 57.0 mL/min (48.5–65.6) resulting in an average half-life ($t_{1/2}$) of 64.2 min (59.9–66.5) (Table 4). Nasal administration of 60 µg exenatide resulted in detectable plasma concentrations with an average C_{max} of 27.6 pg/mL (15.9–46.6), reached (t_{max}) 30 min (15–60) after nasal administration (Fig. 3). These values were in good agreement with models for allometric scaling of exenatide pharmacokinetics [29].

Because only few sampling points with detectable plasma concentrations were observed, extrapolation of AUC values to infinity was less reliable after nasal administration (extrapolated fraction 22%–28%) and, therefore, nasal pharmacokinetics are less well established and give only preliminary indication on accurate

nasal availability of the peptide. However, these data clearly show that nasal absorption is minimal. Nevertheless, the determined volume of distribution of 0.42 L/kg (0.30–0.49), the clearance of 56.6 mL/min (48.5–64.1), and the $t_{1/2}$ of 51.0 min (36.3–67.2) after nasal administration were comparable to those of intravenous exenatide. Absolute nasal bioavailability was calculated at 0.27% (0.16%–0.36%), indicating minimal nasal absorption, which may be explained by the large size of exenatide leading to poor intrinsic membrane penetration.

4. Conclusion

Only few assays for exenatide bioanalysis relying on LC-MS methodologies have been reported with the most sensitive having an LLOQ of 50 pg/mL, hence lacking sensitivity for pharmacokinetic analyses in the therapeutic range. Having an LLOQ of 5 pg/mL (1.2 pM), the presented ultra-sensitive UPLC-MS/MS assay for exenatide quantification in plasma is the first LC-MS-based assay suitable for the pharmacokinetic characterization of exenatide in its intended therapeutic range, which even exhibits superior sensitivity compared to previously reported immunoassays. The assay's dynamic range spans more than three orders of magnitude while concurrently being accurate and precise. The utilization of anion exchange plasma extraction of exenatide performed in a µ-elution format resulted in concentrated extracts, supporting sensitive quantification and rendering the assay suitable for high sample throughput. Using this UPLC-MS/MS assay, absolute nasal bioavailability of native exenatide was found to be very low (bioavailability well below 1%), demonstrating the assay's applicability for pharmacokinetic studies and future formulation development for alternative routes of administration.

Conflicts of interest

The authors declare that there are no conflicts of interest.

References

[1] A.J. Garber, M.J. Abrahamson, J.I. Barzilay, et al., Consensus statement by the American association of clinical endocrinologists and American college of endocrinology on the comprehensive type 2 diabetes management algorithm - 2018 executive summary, Endocr. Pract. 24 (2018) 91–120.
[2] F.K. Knop, A. Bronden, T. Vilsboll, Exenatide: pharmacokinetics, clinical use, and future directions, Expert Opin. Pharmacother. 18 (2017) 555–571.
[3] J. Eng, W. Kleinman, L. Singh, et al., Isolation and characterization of exendin-

4, an exendin-3 analogue, from Heloderma suspectum venom. Further evidence for an exendin receptor on dispersed acini from Guinea pig pancreas, J. Biol. Chem. 267 (1992) 7402–7405.

[4] C.M.B. Edwards, S.A. Stanley, R. Davis, et al., Exendin-4 reduces fasting and postprandial glucose and decreases energy intake in healthy volunteers, Am. J. Physiol. Endocrinol. Metab. 281 (2001) E155–E161.

[5] M.S. Fineman, T.A. Bicsak, L.Z. Shen, et al., Effect on glycemic control of exenatide (synthetic exendin-4) additive to existing metformin and/or sulfonylurea treatment in patients with type 2 diabetes, Diabetes Care 26 (2003) 2370–2377.

[6] L.L. Nielsen, A.A. Young, D.G. Parkes, Pharmacology of exenatide (synthetic exendin-4): a potential therapeutic for improved glycemic control of type 2 diabetes, Regul. Pept. 117 (2004) 77–88.

[7] BYETTA (Exenatide) Injection: US Prescribing Information, AstraZeneca Pharmaceuticals LP, Wilmington, DE, 2018.

[8] BYDUREON (Exenatide Extended-Release) for Injectable Suspension: US Prescribing Information, AstraZeneca Pharmaceuticals LP, Wilmington, DE, 2018.

[9] I. Andrés-Ramos, S. Blanco-Barrios, E. Fernández-López, et al., Exenatide-induced eosinophil-rich granulomatous panniculitis: a novel case showing injected microspheres, Am. J. Dermatopathol. 37 (2015) 801–802.

[10] N.C. Boysen, M.S. Stone, Eosinophil-rich granulomatous panniculitis caused by exenatide injection, J. Cutan. Pathol. 41 (2014) 63–65.

[11] S.C. Jones, D.L. Ryan, V.S. Pratt, et al., Injection-site nodules associated with the use of exenatide extended-release reported to the US Food and Drug administration adverse event reporting system, Diabetes Spectr. 28 (2015) 283–288.

[12] K. Riswold, V. Flynn, Persistent injection site nodules from exenatide: successful treatment with intralesional triamcinolone, JAAD Case Rep. 4 (2018) 830–832.

[13] S.-J. Shan, Y. Guo, Exenatide-induced eosinophilic sclerosing lipogranuloma at the injection site, Am. J. Dermatopathol. 36 (2014) 510–512.

[14] C.I. Vidal, S. Chaudhry, N.M. Burkemper, Exenatide-induced panniculitis: utility of the acid-fast stain to identify injected microspheres, Am. J. Dermatopathol. 40 (2018) 867–869.

[15] M. Fineman, S. Flanagan, K. Taylor, et al., Pharmacokinetics and pharmacodynamics of exenatide extended-release after single and multiple dosing, Clin. Pharmacokinet. 50 (2011) 65–74.

[16] Y.M. Cui, X.H. Guo, D.M. Zhang, et al., Pharmacokinetics, safety, and tolerability of single- and multiple-dose exenatide once weekly in Chinese patients with type 2 diabetes mellitus, J. Diabetes 5 (2013) 127–135.

[17] O.G. Kolterman, D.D. Kim, L. Shen, et al., Pharmacokinetics, pharmacodynamics, and safety of exenatide in patients with type 2 diabetes mellitus, Am. J. Health Syst. Pharm. 62 (2005) 173–181.

[18] P.A. Kothare, H. Linnebjerg, Y. Isaka, et al., Pharmacokinetics, pharmacodynamics, tolerability, and safety of exenatide in Japanese patients with type 2 diabetes mellitus, J. Clin. Pharmacol. 48 (2008) 1389–1399.

[19] J. Malloy, E. Capparelli, M. Gottschalk, et al., Pharmacology and tolerability of a single dose of exenatide in adolescent patients with type 2 diabetes mellitus being treated with metformin: a randomized, placebo-controlled, single-blind, dose-escalation, crossover study, Clin. Therapeut. 31 (2009) 806–815.

[20] FDA Approves First Oral GLP-1 Treatment for Type 2 Diabetes, US Department of Health and Human Services Food and Drug Administration, 2019. https://www.fda.gov/news-events/press-announcements/fda-approves-first-oral-glp-1-treatment-type-2-diabetes.

[21] C. Granhall, M. Donsmark, T.M. Blicher, et al., Safety and pharmacokinetics of single and multiple ascending doses of the novel oral human GLP-1 analogue, oral semaglutide, in healthy subjects and subjects with type 2 diabetes, Clin. Pharmacokinet. 58 (2019) 781–791.

[22] J.F. Zhang, C.J. Sha, Y. Sun, et al., Ultra-high-performance liquid chromatography for the determination of exenatide in monkey plasma by tandem quadrupole mass spectrometry, J. Pharm. Anal. 3 (2013) 235–240.

[23] L.-P. Morin, J.-N. Mess, F. Garofolo, Large-molecule quantification: sensitivity and selectivity head-to-head comparison of triple quadrupole with Q-TOF, Bioanalysis 5 (2013) 1181–1193.

[24] J.R. Kehler, C.L. Bowen, S.L. Boram, et al., Application of DBS for quantitative assessment of the peptide Exendin-4; comparison of plasma and DBS method by UHPLC–MS/MS, Bioanalysis 2 (2010) 1461–1468.

[25] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services Food and Drug Administration, 2018. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf.

[26] Guideline on Validation of Bioanalytical Methods, EMEA/CHMP/EWP/192217/2009, European Medicines Agency, 2009. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/00/WC500109686.pdf.

[27] B. Matuszewski, M. Constanzer, C. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC– MS/MS, Anal. Chem. 75 (2003) 3019–3030.

[28] M. Sauter, P. Uhl, M. Majewsky, et al., An ultrasensitive UPLC–MS/MS assay for the quantification of the therapeutic peptide liraglutide in plasma to assess the oral and nasal bioavailability in beagle dogs, Bioanalysis 11 (2019) 887–898.

[29] T. Chen, D.E. Mager, L. Kagan, Interspecies modeling and prediction of human exenatide pharmacokinetics, Pharm. Res. (N. Y.) 30 (2013) 751–760.