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Determination of Taspoglutide in Human and Animal Plasma Using Liquid Chromatography−Tandem Mass Spectrometry with Orthogonal Column-Switching

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A highly sensitive liquid chromatographic method with online solid-phase extraction (SPE) and tandem mass spectrometric detection was developed for the quantification of the synthetic peptide drug taspoglutide in human and animal plasma. Sample preparation consisted of simple protein precipitation or automated off-line SPE using mixed mode cation exchange material, optionally preceded by cleavage of potential antidrug antibody complexes. Excellent selectivity was obtained by a novel orthogonal column switching approach, employing hydrophilic interaction liquid chromatography (HILIC) for online SPE followed by chromatographic separation on a 300 Å pore size C18 column. The use of a stable isotope labeled drug (C-terminal arginine containing six ^{13}C and four ^{15}N atoms) as internal standard improved the quality and ruggedness of the method. The lower limits of quantitation (LLOQ) in human and animal plasma were 10.0 and 50.0 pg/mL, respectively, extracting only 250 or 100 μL sample aliquots. Precisions and accuracies were below 15% CV and between 89% and 114%, respectively. The method was successfully employed to analyze samples from pharmacokinetic studies.

Pharmaceutical companies started more than 10 years ago to complement their small molecule portfolios with biomolecules. Although they have often high systemic clearance and low oral bioavailability, they show higher specificity and are less prone to toxicological and drug-drug interaction liabilities than small molecule drugs. Recent advances in synthetic optimization of pharmacological and pharmacokinetic properties and manufacturing improvements have contributed to the growing market share of therapeutic proteins and peptides.[1−4] Taspoglutide, a human glucagon-like peptide-1 (GLP-1) analogue with a molecular weight of 3340, is currently in development for the treatment of type 2 diabetes.[5] It is a compound similar to the native hormone GLP-1 which has a key role in blood sugar regulation.[6] The high potency (therapeutic dose below 1 mg/kg subcutaneously) and thus resulting low drug levels in biological samples demand extremely sensitive bioanalysis. Another challenge which has to be considered during bioanalytical method development is the potential immunogenicity of taspoglutide: repeated treatments with the drug can lead to formation of antidrug antibodies (ADAs).[4] Hence, the drug is circulating in the body as free form, protein bound, and also bound to neutralizing or non-neutralizing ADAs. To determine the total drug concentration, dissociation of drug-ADA bonds is required. Quantitation of biomolecules in biological fluids is most often performed using immunological methods, such as enzyme-linked immunosorbent assay (ELISA).[7] The high sensitivity and specificity for the target analyte are today still unmatched by other techniques. However, immunoassays were not an option for taspoglutide analysis because of the long development time, need for expensive antibodies, and potential interferences from endog-

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enous compounds. Liquid chromatography coupled to electrospray ionization and mass spectrometric detection (LC-ESI-MS/(MS)) has become a powerful tool for quantitation of therapeutic peptides[8−18] and proteins[19−26] in biological matrixes. Its advantages over ELISA are the rapid method development, possibility to distinguish between drug and its metabolites and interferences from the matrix, feasibility of cocktail analysis, and wider dynamic range. However, extensive sample cleanup and enrichment is usually needed for high sensitivity, and the throughput may be limited by long chromatographic run times which are often necessary for good selectivity. Small protein and peptide drugs with a MW below 10.000 Da were usually determined as intact molecules employing isotopically labeled[12,16,26] or structurally related analogues[11,14,20] as internal standards, whereas large molecules required protein digestion before LC-MS analysis.[21−24] Dissociation of drug-ADA bonds was performed through addition of acids or guanidine before sample extraction.[20,26] As guanidine totally unfolded mAbs and thus destroyed most protein−protein interactions occurring between antibody and antigen, it was reported as a very efficient dissociating agent.[27] Various sample preparation methods have been used to extract protein and peptide drugs from biological matrixes, including (1) simple and fast protein precipitation (PP),[12,16,19] (2) more elaborate but efficient solid-phase extraction (SPE) using reversed phase (RP) or ion exchange material,[13,22,25] or (3) specific but time-consuming and laborious immunoaffinity extraction.[24] Online SPE provided an automated cleanup procedure but at the expense of prolonged run time.[18] For the subsequent LC separations, careful selection of mobile phase composition was essential to obtain good sensitivity and separation efficiency.[8,9,28] Conventional RP columns were mostly used for biomolecule drug candidates, whereas biomarker analysis also employed micro-LC,[29] nano-LC,[30] LC using sub-2 μm particles,[31,32] or two-dimensional LC.[33−35] The highest orthogonality, and thus enhanced selectivity, was achieved in 2D chromatography when combining hydrophilic interaction liquid chromatography (HILIC) with RP separations.[35,36] Mass spectrometric detection was usually performed on triple quadrupole instruments, operating in selected reaction monitoring (SRM) mode. Several authors preferred the single ion monitoring (SIM) mode for determination of peptide drugs,[11,15,37] biomarkers,[33−35] or hormones.[29] Field-asymmetric waveform ion mobility mass spectrometry (FAIMS) has been used to reduce chemical background and filter out isobaric interferences, hence increasing the signal-to-noise (S/N) ratio.[14,15,37] Quantitation limits for small proteins and peptides in the above-described methods were mainly in the high pg/mL to low ng/mL range (approximately 1 order of magnitude higher for large molecules), and only few more sensitive methods were reported. LLOQs between 10 and 50 pg/mL were achieved for peptides with MWs below 650 using PP and SPE, followed by SRM-LC-MS/MS with a 50 min run time.[13] A sensitive method for native GLP-1 in human plasma with an LLOQ of 20 pg/mL employed immunoextraction of 1 mL sample, followed by a 35 min micro-LC run and single MS detection.[29] Taspoglutide has been previously analyzed in plasma by LC-MS/MS already, involving off-line solid-phase extraction (SPE), followed by online SPE on a Symbiosis system and LC-MS/MS using an API 4000 instrument (unpublished method developed at Ipsen Pharma). A 1.5-mL aliquot of human plasma per analysis was required to achieve an LLOQ between 12.5 and 50.0 pg/mL, and cleavage of drug-ADA bonds was not performed. Aliquots of 200 μL animal plasma were extracted after drug-ADA cleavage with guanidine to obtain an LLOQ of 100 pg/mL. To support phase III clinical trials, we developed a more robust, sensitive and efficient LC-ESI-MS/MS method with an LLOQ of 10 pg/mL using only 250 μL of human plasma. Sample preparation comprised an incubation step to dissociate reliably possible drug-ADA complexes, followed by simple protein precipitation. To achieve high selectivity we combined HILIC with RP C18 separations. The total run time was 7.2 min. Stable isotope labeled taspoglutide served as internal standard (IS)

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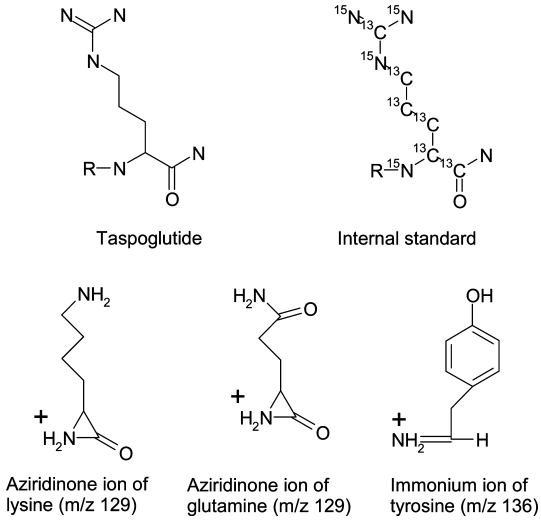

Figure 1. Structures of taspoglutide (drug) and internal standard (IS), R = His-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Aib. Structures of monitored fragment ions.

throughout the entire analysis procedure. Animal plasma was analyzed after off-line SPE, using only a 100 μL sample to obtain an LLOQ of 50.0 pg/mL. The method was validated according to regulatory guidelines for bioanalytical method validation of chromatographic assays for small molecules[38,39] and employed to analyze samples from pharmacokinetic studies.

EXPERIMENTAL SECTION

Materials. The peptide drug taspoglutide, MW 3339.7 g/mol, and the $^{13}C_6{}^{15}N_4$ labeled IS, MW 3347.7 g/mol, were synthesized at F. Hoffmann-La Roche. The structures are shown in Figure 1. Ethanol, methanol, and isopropanol (Lichrosolv for HPLC) were obtained from Merck (Darmstadt, Germany), and acetonitrile (HPLC grade S) from Rathburn (Walkerburn, U.K.). Ammonium formate (p.a.), formic acid 98−100% (Suprapur grade), acetic acid (100%, p.a.), phosphoric acid (85%, p.a.), and ammonium hydroxide were purchased from Fluka (Buchs, Switzerland). Guanidine HCl was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Aprotinin solution (10000 kallikrein inhibitory units, KIU/mL) was purchased from Bayer (Zurich, Switzerland). The water used for the preparation of all solutions was obtained from a Milli-Q apparatus (Millipore, Billerica, MA, U.S.A.). Polypropylene tubes (1.5 mL) and low-bind 96-deep-well plates (DWPs) were obtained from Eppendorf (Hamburg, Germany), 96-DWP from Milian Instruments (Geneva, Switzerland). Oasis MCX 96-well plates 30 μm, 30 mg were purchased from Waters (Milford, MA, U.S.A.). Blank EDTA human plasma was purchased from a blood bank (TRINA Bioreactives, Nänikon, Switzerland). Blank EDTA animal plasma was supplied by laboratories at F. Hoffmann-La Roche. Aprotinin solution was added to plasma to obtain a final concentration of 100 KIU/mL plasma (corresponding to 50 KIU/mL blood, assuming that 1 mL blood produced 0.5 mL plasma). Rabbit serum containing 3 mg-eq/mL anti-taspoglutide antibody, used to mimic drug-ADA binding in real samples, was obtained from Ipsen Pharma (Barcelona, Spain).

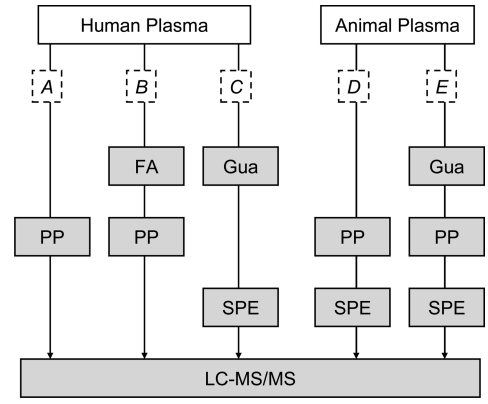

Figure 2. Sample preparation workflow. A to E = sample preparation methods, FA = ADA-drug dissociation using formic acid, Gua = ADA-drug dissociation using guanidine, PP = protein precipitation, SPE = solid phase extraction. A and D used in absence of ADAs, B and E used in potential presence of ADAs, C optionally used in presence of ADAs.

Preparation of Standards and Quality Controls. Stock solutions of the drug and IS were prepared in 0.1 M acetic acid at 1 mg/mL. The stock solutions were stable at −70 °C for at least 4 months. The IS stock solution was diluted with ethanol/water/formic acid 90:10:0.1 (v/v/v) to obtain a working solution at 10 ng/mL which was stable at 4 °C for at least 3 months. The drug stock solution was spiked to human blank plasma to obtain a working solution at 5 μg/mL plasma; this solution was serially diluted with blank plasma, yielding concentrations between 10 pg/mL and 5 ng/mL for human plasma and between 50 pg/mL and 25 ng/mL for animal plasma. Quality control (QC) samples at three concentrations were prepared by the same procedure. Additional QC samples at 100 pg/mL and 4 ng/mL were prepared in human plasma spiked with rabbit antiserum to obtain 5 μg/mL antibody and incubated for 60 min at 37 °C; these QCs were used to evaluate the capacity of the method to release the drug from possible ADAs generated in real samples. Previous data (unpublished) indicated that calibration standards and QCs were stable at room temperature for 72 h, at −20 °C for 30 months and for six freeze-thaw cycles.

Extraction Procedures for Human Plasma. A sample preparation flowchart is shown in Figure 2. Method A was employed if no dissociation of drug-ADA bonds was required (e.g., in absence of ADAs or for tests of binding), whereas methods B and C incorporated two different options for the cleavage of drug-ADA bonds, either with formic acid or guanidine.

Method A. To aliquots of 250 μL of plasma were added 20 μL of internal standard working solution and 1 mL of ethanol/acetonitrile/formic acid 30:50:0.1 (v/v/v) to obtain protein precipitation, followed by mixing and centrifugation.

Method B. To aliquots of 250 μL of plasma were added 20 μL of internal standard working solution and 75 μL of formic acid (4% in acetonitrile) for dissociation of drug-ADA bonds, well mixed and incubated for 2 h at room temperature. Aliquots of 1 mL of ethanol/acetonitrile/formic acid 30:50:0.1 (v/v/v) were then added to the sample to obtain protein precipitation, followed by mixing and centrifugation.

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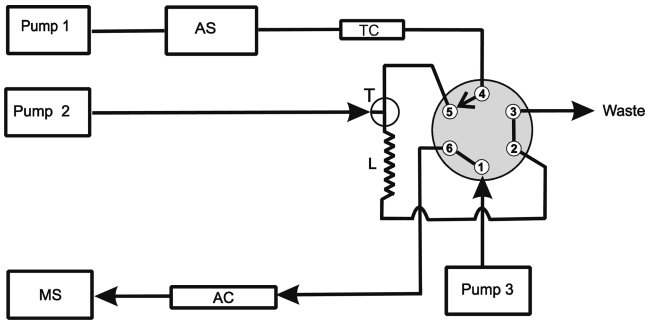

Figure 3. Scheme of column-switching LC-MS/MS system. AS = autosampler, TC = trapping column, AC = analytical column, MS = mass spectrometer. Valve in position A.

Method C. To aliquots of 500 μL of plasma were added 20 μL of internal standard working solution and 500 μL of 6 M aqueous guanidine solution for dissociation of drug-ADA bonds, followed by addition of 100 μL ethanol, and the mixture was incubated at 45 °C for 30 min. Optionally, 500 μL of water were added instead of guanidine as a positive control of ADA binding. After addition of 100 μL 0.5% phosphoric acid, the sample solution was loaded onto an Oasis MCX 96-well plate which was preconditioned with methanol and 0.5% formic acid in water/isopropanol 9:1 (v/v). The plate was washed with 0.5% formic acid in water/isopropanol 9:1 (v/v), then with methanol/2% aqueous formic acid 96:4 (v/v), and finally with acetonitrile/2% aqueous formic acid 96:4 (v/v). Aliquots of 500 μL solvent were used in all preconditioning and rinsing steps. The analyte and internal standard were eluted with two 200 μL portions of acetonitrile/ethanol/water/25% aqueous ammonium hydroxide 24:7:7:2 (v/v/v/v). To this solution were added 600 μL of acetonitrile/ethanol/water/formic acid 30:24:5:1 (v/v/v/v).

Extraction Procedure for Animal Plasma. *Method D.* A combination of PP and SPE was employed in absence of ADAs. To 100 μL plasma was added 20 μL of internal standard working solution. After addition of 1 mL methanol and centrifugation, 200 μL of 20% aqueous formic acid were added, and the sample was loaded onto the Oasis MCX plate, followed by the same washing and elution steps as for human plasma (method C).

Method E. This method was used for cleavage of drug-ADA bonds. After adding the IS, incubation with 100 μL 6 M guanidine solution was performed for 30 min at 45 °C, and PP and SPE were carried out as described for method D.

Pipetting of samples, addition of internal standard, and all transfer steps of samples and solvents were performed with a Tecan Genesis RSP 100/4 liquid handler (Tecan Schweiz AG, Männedorf, Switzerland). A 96-plate vacuum manifold (Phenomenex, Torrance, U.S.A.) mounted on the Genesis together with a membrane pump (KNF Neuberger, Balterswil, Switzerland) was used during SPE.

Chromatographic Conditions. A schematic representation of the HPLC column-switching system is given in Figure 3. The HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland) was equipped with a 1000 μL sample loop. Needle and valve rinse between injections was performed using water/ethanol 90/10 (v/v) and ethanol. A 1200-series quaternary pump (pump 1; Agilent Technologies, Waldbronn, Germany) delivered mobile phase A1 (water/methanol/aqueous 1 M ammonium formate/formic acid, 500:500:12.5:5, v/v/v/v) or B1 (water/acetonitrile/methanol/1 M ammonium formate/formic acid, 50:700:250:12.5:5, v/v/v/v/v). The dilution pump (pump 2) was a LC20AT (Shimadzu, Kyoto, Japan) controlled by a CBM-20A module and delivered mobile phase A2 (water/1 M ammonium formate/formic acid, 993:5:2, v/v/v). The trapping valve V (electrically driven 6-port 2-position valve, VICI Valco, Houston, TX, U.S.A.) was equipped with a 500 μL loop which was connected via a T-piece with the dilution pump. An Agilent 1200-series binary pump (pump 3) delivered mobile phases A3 (water/1 M ammonium formate/formic acid, 988:10:2, v/v/v) and B2 (water/acetonitrile/1 M ammonium formate/formic acid 88:900:10:2, v/v/v/v). The internal mixer and pulse dampener were bypassed to reduce the dead volume of the pump. The trapping column (TC) was a 50 × 2.1 mm Atlantis HILIC Si, 3 μm with precolumn filter (Waters). The analytical column (AC) consisted of two 75 × 2 mm Zorbax Poroshell 300-SB C18, 5 μm columns (Agilent) in series. The trapping column was maintained at 20 °C and the analytical column at 80 °C in the Agilent column oven. The time program for online SPE and chromatographic separation with total cycle time of 7.2 min is shown in Table 1. An aliquot from the sample extract (800 μL) was injected onto the trapping column (TC), and endogenous lipophilic compounds were rinsed off while the analyte and the internal standard were retained. Analyte and IS were then eluted into the loop while polar compounds remained on the TC. Because of the dead volume of 800−1100 μL of pump 1, the analyte and IS reached the loop at 4.5 min. At 4.7 min, the loop was decoupled from the TC and connected to the AC, and the analyte and IS were transferred in back-flush mode to the AC. As soon as the transfer was completed, loop and AC were disconnected, followed by separation on the AC in isocratic mode using a flow gradient. The dilution pump served to dilute the eluent from the TC before entering the loop and again during transfer onto the AC.

Table 1. LC Time Program

time (min)	component	mobile phase	flow rate (mL/min)	valve position
0	pump 1	10%A1/90%B1	0.6	
	pump 2	100%A2	0.1	
	pump 3	100%B3	0.6	
	valve	n/a	n/a	position A
0.55→0.65	pump 1	A1/B1 10:90→40:60	0.6	
1.0→1.1	pump 3	100%B3→A3/B3 63:37	0.6	
2.1→2.6	pump 1	A1/B1 40:60→10:90	0.6	
3.4→3.5	pump 2	100%A2	0.1→0.4	
4.7	valve	n/a	n/a	position B
5.0→5.1	pump 1	A1/B1 10:90	0.6→0.8	
5.2→6.4	pump 3	A3/B3 63:37	0.6→1.0	
5.3	valve	n/a	n/a	position A
5.4→5.5	pump 2	100%A2	0.4→0.1	
5.9→6.2	pump 1	A1/B1 10:90	0.8→1.2	
6.7→6.8	pump 3	A3/B3 63:37	1.0→0.6	
6.9→7.0	pump 3	A3/B3 63:37→100%B3	0.6	
7.1→7.2	pump 1	A1/B1 10:90	1.2→0.6	

Mass Spectrometric Conditions. The mass spectrometer was an API 5000 (ABI/MDS Sciex, Concord, ON, Canada) equipped with a turbo ion spray interface maintained at 650 °C. The interface heater was turned on, the entrance potential was set to 10 V, and the electrospray voltage was maintained at 5500 V. Nitrogen was used as nebulizing, auxiliary, curtain and collision gas with instrument settings of 60, 50, 18, and 5, respectively. The mass spectrometer was operated in the positive ion SRM mode. The selected mass-to-charge ratios (m/z) for quantitation were 835.9→136.2 for the drug and 838.4→129.0 for the IS. The collision

energies were optimized at 75 and 89 eV for drug and IS, respectively. Data acquisition was carried out at unit resolution (peak width at half-height set to 0.7 amu in Q1 and Q3). The dwell time was 200 ms. To monitor the matrix effect caused by phospholipids, precursor ion scans of m/z 184 were carried out between m/z 350 and 800, setting the collision energy at 25 eV. The Analyst 1.4.2 software was used for instrument control, data acquisition, and integration.

Method Validation. The selectivity and specificity of the method was investigated by analysis of blank human plasma from six different donors and blank animal plasma from at least three different pooled batches. Isobaric endogenous compound peaks at the retention time of taspoglutide should be below 20% of the analyte peak at the LLOQ (defined here as the minimum concentration that yielded a S/N ratio of at least five and that could be measured routinely with a precision of lower than 20% and an inaccuracy of lower than 20%). The calibration curves were considered as linear if the deviations from nominal concentrations were below 20% at LLOQ and below 15% at all other levels. Linear regression with $1/x^2$ weighing was employed. Interassay precision and accuracy were determined by independent analysis of five sets of QC samples (each $n = 1$) against five calibration curves in independent runs; intra-assay data resulted from 5-fold analysis of QC samples against one calibration curve in the same run. The recovery was investigated in human and rat plasma for three concentrations comparing the response of taspoglutide in spiked and extracted plasma samples with post-extraction spiked blank plasma extracts (denoted as "reference samples"). The recovery results were based on the peak area ratios analyte/IS; the IS was spiked after extraction to correct only for variability during LC-MS/MS analysis. The matrix factor was investigated comparing the analyte responses in reference samples and in solutions containing taspoglutide at the nominal concentration in matrix-free solvent. The absolute matrix factor data were calculated using peak areas, and the relative matrix factors were calculated using area ratios analyte to IS according to a recommended procedure.[39] In routine analysis, the acceptance criteria for regulatory bioanalysis recommended by the FDA[38] were applied for human samples, whereas less stringent criteria were employed in the animal formulation studies (less than 20% CV and ±20% deviation from accuracy). The criteria for successful cross-validation were based on the suggestions for incurred samples reanalysis:[39] two-thirds of the results should be within 20% of the mean.

Pharmacokinetic Studies. Taspoglutide was subcutaneously administered to rats, cynomolgus monkeys, and minipigs (1 to 20 mg/animal). Blood samples (up to 696 h postdose) were collected into EDTA tubes containing aprotinin at 50 KIU/mL blood, followed by centrifugation for plasma separation. Human plasma samples from 20 healthy volunteers were obtained from a clinical study, in which repeated doses of 20 mg taspoglutide were administered by subcutaneous injection of a sustained release formulation. Blood was collected into EDTA/aprotinin tubes up to 84 days postdose. Cross-validation between non-dissociating and acid dissociating assay was performed, analyzing 15 spiked QC samples and 15 pooled human study samples (antibody negative) with both assays. Cross-validation between acid and guanidine dissociation was carried out by the analysis of 60 human plasma samples from 5 subjects (antibody positive and negative).

RESULTS AND DISCUSSION

To achieve low limits of quantification, a sensitive mass spectrometer (API 5000) was used, large sample aliquots were injected, and the analyte was separated from matrix compounds to prevent signal suppression. Depending on the sample matrix, simple protein precipitation or off-line SPE were performed. The release of drug bound to ADA was optimized. The necessary selectivity was obtained by orthogonal HILIC to C18 column switching and detection in SRM mode. Details on development and performance of the method are presented in the following sections.

General Remarks. Taspoglutide binds strongly onto surfaces, resulting in apparent instability in neat solution (because of adsorption effects of particularly low drug concentrations), substance loss during sample preparation, and carry-over in the LC system. Because of high protein binding, taspoglutide is well stabilized in plasma, and therefore, calibration standards and quality control samples were prepared by serial dilution of plasma spiked at a high concentration with blank plasma. An organic solvent content of at least 30% was maintained in sample solutions and mobile phases to keep the drug in solution. During sample preparation, evaporation and reconstitution steps were avoided to prevent compound loss. Rinsing the trapping column between injections with a mixture of methanol and acetonitrile decreased the carry-over to less than 0.5% of the signal from the previous injection. This remaining carry-over was taken into consideration during the setup of the sample order in analytical run sequences.

Development of MS Conditions. Three-, four-, and five-fold protonated molecular ions, which fragmented to a large number of product ions, were observed in Q1 for drug and IS. Full-scan Q1 and product ion spectra for taspoglutide are shown in Figure 4. The fragmentation patterns of drug and IS were identical. Any specific product ions above 1200 amu were not detectable because of the limited mass range of the API 5000 instrument. The low-mass fragment ions resulting from amino acids ions (for structures see Figure 1) were not specific for the peptide drug, whereas the more drug specific fragments with higher masses were less intense. To find the optimum transition regarding sensitivity and selectivity, four to five of the most intense fragments were selected for each of the three-, four-, and five-fold charged precursor ions, and the signal intensities and co-eluting endogenous compounds in blank plasma were compared in SRM LC-MS/MS. Although the finally selected transition at m/z 835.9→136.2 for taspoglutide did not produce the most intense signal, it showed the least endogenous interferences eluting at or close to the retention time of the drug. Because of interfering peaks at m/z 136.2 the IS was monitored at m/z 838.4→129.0. Higher mass fragments, for example, at m/z 960.4, although being drug specific, did not provide sufficient intensity even after optimizing the collision energy (47 eV) and gas pressure. The SIM mode (e.g., at m/z 835.9) showed a 10-fold higher absolute signal intensity but was not an alternative because of the 20-fold lower S/N ratio and larger number of interfering compounds compared to SRM. Higher resolution at Q1 or Q3 did dramatically reduce the signal, thus no gain in S/N ratio was observed.

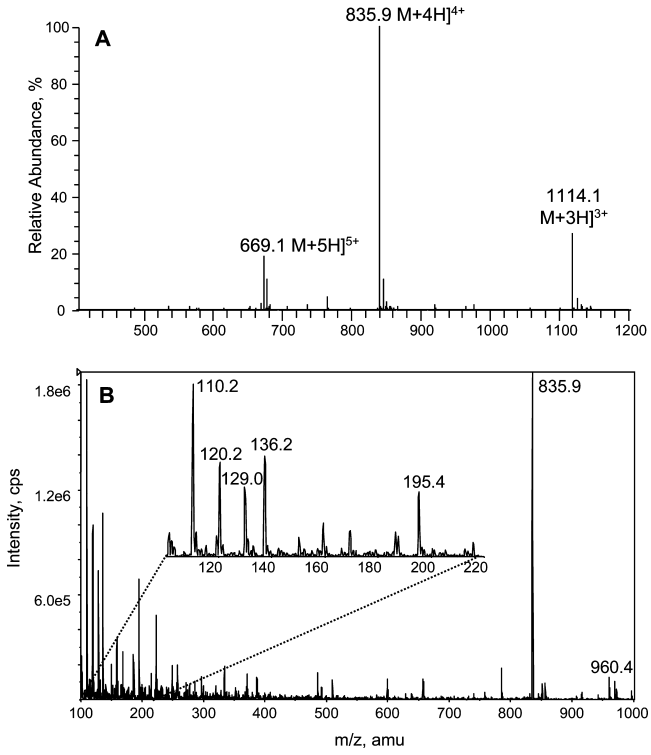

Figure 4. Q1 full-scan spectrum of taspoglutide (A) and product ion spectrum (MCA: accumulated spectrum during CE ramping from 5 to 130 eV) of precursor *m*/*z* 835.9 (B).

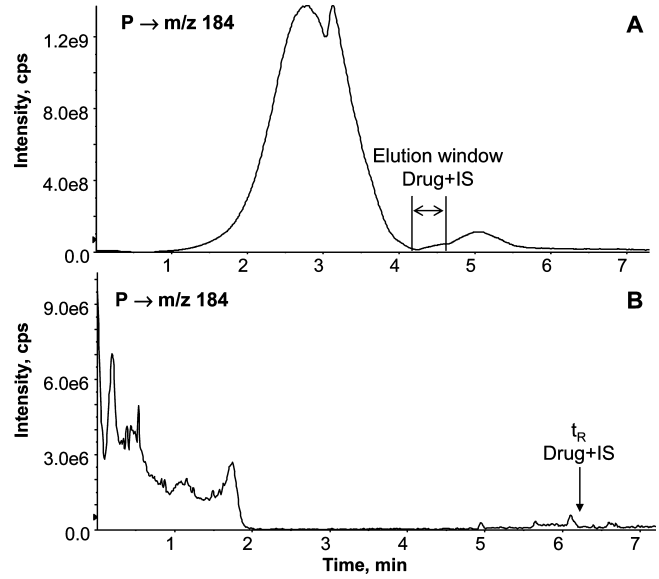

Figure 5. Visualization of matrix effect caused by phospholipids: chromatograms from precursor ion (P) scans of product *m*/*z* 184 after direct elution from trapping column to MS (A) and after complete trapping cycle with analytical separation (B).

Development of Chromatographic Conditions. The HILIC trapping column (TC) was ideal for online SPE followed by RP chromatography because (1) large volumes of highly organic sample solutions could be directly applied without prior dilution, (2) the polar analyte and IS were selectively enriched while lipophilic sample constituents were rinsed off, and (3) increased selectivity via an orthogonal mechanism was provided[36] (in contrast to a combination of two RP columns which resulted in a significant higher number of matrix components interfering with the analyte peak, data not shown here). Elution from TC was carried out with an increased aqueous solvent content which was compatible to the subsequent RP separation. Online dilution of the sample plug during transfer from TC onto the analytical column (AC) was necessary to decrease the elution strength and thus provide sufficient retention of taspoglutide. Placing a loop between TC and AC for parking the analyte peak had the advantages of (1) independently optimizing the trapping and analytical conditions, (2) providing a pseudo heart-cut as in 2D-LC by transferring only a small portion of the eluate from trapping onto analytical column, and (3) avoiding a substantial increase in backpressure when coupling the columns directly. Among various analytical columns tested during method optimization, the Poroshell C18 AC provided the most efficient separation from closely eluting isobaric endogenous compounds, although the peak shape was compromised. Two columns of 75 mm length were coupled to increase the resolution (a longer column was not available). The mobile phase, containing ammonium formate and formic acid, was selected to balance between signal intensity, peak shape, and resolution. Isocratic elution was employed because organic solvent gradients led to the elution of endogenous interferences close to or at the retention time of the analyte and internal standard. A flow gradient provided improved peak shape while still maintaining sufficient separation from isobaric matrix compounds. Heating the AC to 80 °C was mandatory to obtain symmetrical, narrow, and intense peaks. Before use, the AC was conditioned with 1% TFA in water/acetonitrile/methanol 40:30:30 overnight. This conditioning step was repeated in regular time intervals (usually every 3 weeks, after about 3000 plasma injections) to maintain the column performance. The orthogonal HILIC to RP separation mechanism led to efficient removal of matrix compounds, which was demonstrated by monitoring the elution of phospholipids. Phospholipids are known as the main compound class that causes ionization effects (mainly signal suppression) in mass spectrometric detection.[40] Their removal was monitored during method optimization by running a precursor ion scan of a common fragment at *m*/*z* 184. As shown in Figure 5A, a major part of the phospholipids is rinsed off the TC, and only a small amount is transferred together with drug and IS onto the AC, where they were well separated (Figure 5B).

Optimization of Sample Extraction. Protein precipitation (PP) was employed successfully because of the relatively small size of the peptide drug. In combination with the subsequent column-switching LC-MS/MS procedure, it was well suited for human plasma. The ratio plasma/solvent was optimized at 1:4 (v/v) to provide sufficient removal of matrix proteins while avoiding co-precipitation of taspoglutide. The organic solvent composition of ethanol-acetonitrile 3:5 (v/v) was selected to obtain a high recovery from plasma and to prevent break-through on the HILIC trapping column when injecting large sample volumes. Formic acid was added because acidic conditions enhanced the trapping efficiency. PP could not be employed after guanidine treatment to cleave drug-ADA bonds (see section Cleavage of Drug-ADA Bonds) because a substantial number of interfering endogenous compounds were observed during MS/MS detection.

(40) Ismaiel, O. A.; Halquist, M. S.; Elmamly, M. Y.; Shalaby, A.; Karnes, H. T. *J. Chromatogr. B* **2007**, *859*, 84–93.

Table 2. Recovery[a] of Taspoglutide (%) from Human Plasma Containing 5 μg/mL Rabbit ADA after Sample Preparation without and with Drug-ADA Cleavage (n = 2)

ADA cleavage	sample preparation method[b]	100 pg/mL	4000 pg/mL
no	SPE	16	30
	PP	40	24
yes	Urea (6 M)/plasma 1:1, 30 min, 45 °C; SPE	19	35
	Guanidine (6 M)/plasma 1:1, 30 min, 45 °C; SPE	94	96
	Formic acid (2% in ACN)/plasma 1:3, 2 h, RT; PP	~80	~85
	Formic acid (4% in ACN)/plasma 1:3, 2 h, RT; PP	96	99

[a] [Peak area ratio analyte/IS in antibody QC/peak area ratio in regular QC] × 100%, not corrected by IS since IS added after extraction. [b] SPE: solid-phase extraction; PP: protein precipitation; RT: room temperature; ACN: acetonitrile.

Therefore, an off-line SPE method was developed. MCX material was chosen because of best recovery and least remaining endogenous interferences compared to other SPE materials. Phosphoric acid was added to the human plasma samples before application onto the SPE plate to reduce viscosity. To obtain a high recovery of analyte and IS it was necessary to precondition the SPE material first with methanol and then with water/isopropanol 90/10 (v/v). Furthermore it was important to maintain an organic solvent content of at least 10% in all solutions applied to the stationary phase. Sample loading and elution was performed by gravity flow because the application of vacuum or positive pressure resulted in significantly lower recoveries. Washing with both acidic acetonitrile and methanol was essential to effectively remove endogenous compounds which would interfere during subsequent LC-MS/MS analysis. Animal plasma required a combination of PP and subsequent SPE to effectively remove matrix components which otherwise co-eluted with the analyte and caused interfering peaks or severe ionization suppression. As the elution from the SPE plates was performed at basic conditions, the final injection solution had to be neutralized by adding formic acid; the ratio of solvents (ethanol/acetonitrile/water) was optimized to prevent breakthrough on the HILIC trapping column.

Cleavage of Drug-ADA Bonds. In our experiments to optimize the release of taspoglutide from any potential drug-ADA complexes, rabbit antiserum was used to mimic ADAs which are formed in real samples. The efficiency to cleave drug-ADA bonds was investigated by comparing the response in QC samples with and without ADA at different dissociation conditions. The results are shown in Table 2. Positive controls (QCs without addition of a dissociating agent) demonstrated that the rabbit antibody actually bound to the peptide drug, and could not be released completely during PP (extraction method A) or SPE (without cleaving agent). Urea was not suited to release the drug from ADA complexes, whereas the drug was quantitatively recovered after incubation with guanidine for 30 min at 45 °C. Incubation with acetonitrile containing 2% formic acid according to a previously described procedure[22] did not quantitatively dissociate the drug-ADA bonds, and the drug release was not reproducible in repeated experiments. Increasing the formic acid content to 4% did improve the dissociation, with the advantage of reducing the subsequent sample preparation efforts by applying simple protein precipitation instead of SPE. However, in the case of real plasma samples containing spontaneous anti-taspoglutide antibodies the total release of the drug from these antibodies can not be fully guaranteed as the antigen−antibody binding might be different from the spiked samples. Guanidine is recognized as a very powerful cleaving agent[27] and was proven to be efficient for ADA-positive animal samples (previous experiments performed at Ipsen Pharma, unpublished). Therefore, both the acid and the guanidine treatment (extraction methods B and C) were validated together with the LC-MS/MS method for optional use during study sample analysis. In case of high ADA levels the guanidine treatment may be used to confirm data obtained with the acid dissociation.

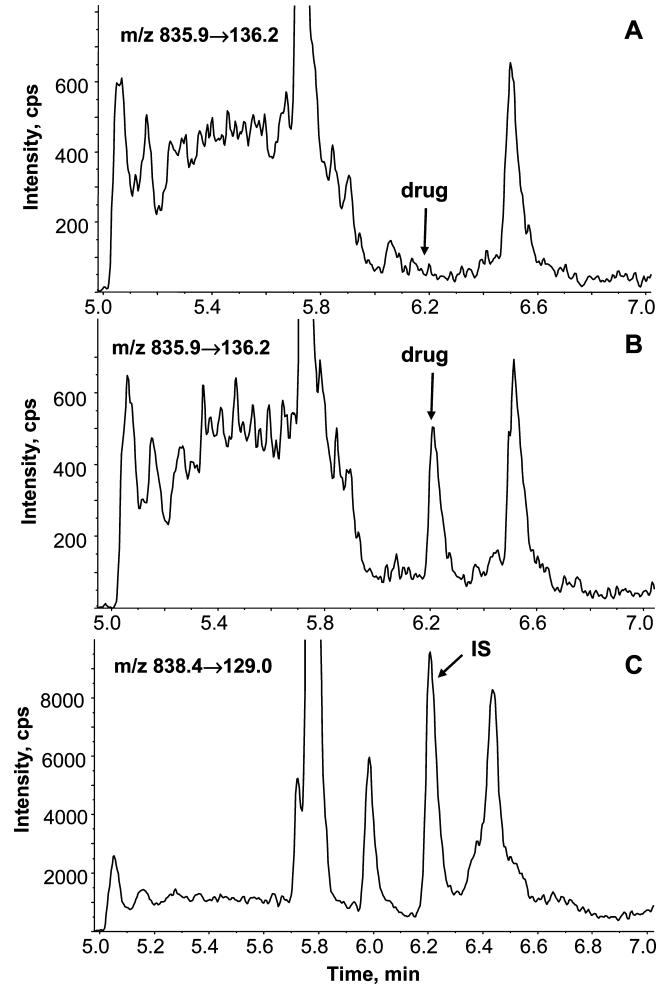

Figure 6. SRM chromatograms of drug taspoglutide in human double blank plasma (A) and in human plasma spiked at LLOQ 10.0 pg/mL (B) and of internal standard in human plasma (C). Sample preparation by PP after acidic dissociation.

Assay Performance. No endogenous interferences were present at the retention time of taspoglutide. Representative chromatograms of double blank human and rat plasma are shown in Figures 6 and 7. The LLOQ was 10.0 pg/mL for human and

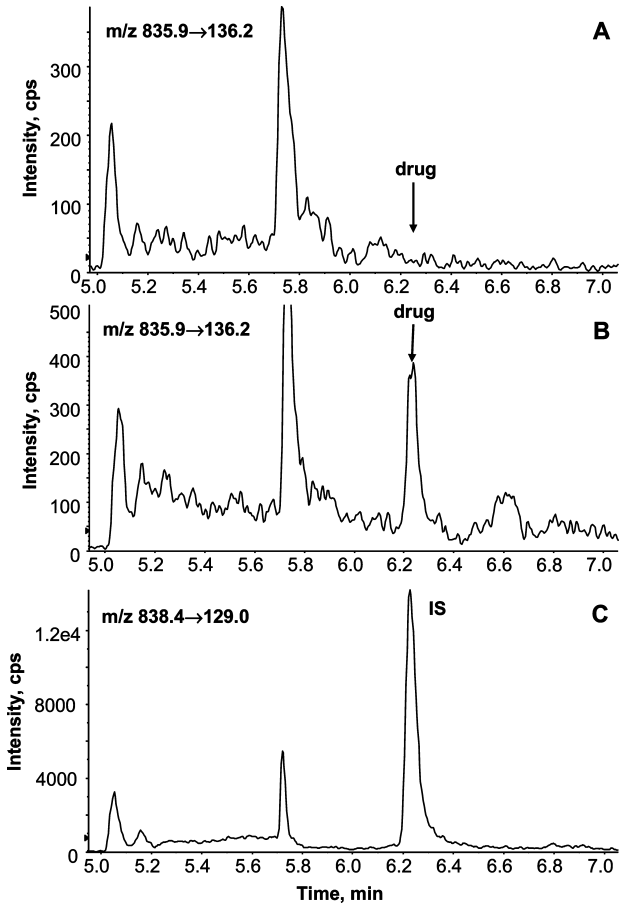

Figure 7. SRM chromatograms of drug taspoglutide in rat double blank plasma (A) and rat plasma spiked at LLOQ 50.0 pg/mL (B) and of internal standard in rat plasma at LLOQ (C). Sample preparation by PP and SPE after guanidine dissociation.

50.0 pg/mL for animal plasma (see Figures 6 and 7 for typical chromatograms of the lowest calibration standards in human and rat plasma). The calibration functions were linear over 3 orders of magnitude from 10.0 pg/mL to 5000 pg/mL for human plasma and from 50.0 pg/mL to 25000 pg/mL for animal plasma. Linear least-squares regression with $1/x^2$ weighing described best the concentration-response relationship. Mean relative errors of calibration standards in human plasma during inter-assay validation ranged between 0.1% and 4.1%, the average slope of the calibration curves was 0.00447 (10.5% RSD), and the intercept values corresponded to less than 10% of the area ratio at LLOQ. Inter-day precision and accuracy data obtained from QC samples in human plasma are compiled in Table 3. Intra-day validation produced comparable precisions and accuracies (data not shown). Table 4 shows precision and accuracy data in rats; similar results were obtained for dog, cynomolgus monkey, and minipig plasma, with precisions less than 11.0% CV and accuracies between 95.0% and 114.1%. A previously used structural analogue did not co-elute with taspoglutide during off-line SPE and chromatography and could therefore not compensate for matrix suppression and recovery variability (data not presented), but the stable isotope labeled analogue employed as IS enhanced the assay quality and ruggedness. Recovery and matrix factor data are compiled in Table 5. As expected, a somewhat lower (but overall satisfactory) recovery was obtained when using SPE instead of PP for human plasma. Because of the cleaner extracts obtained from SPE, no significant matrix effect was observed. Slight ionization suppression occurred after PP because of remaining matrix components; however, the IS compensated this effect.

Table 3. Inter-Day Precision and Accuracy of Taspoglutide Quality Control Samples in Human Plasma (n = 5)

sample preparation	added concentration (pg/mL)	precision (%)	accuracy (%)
PP[a]	10	10.3	103.6
	20	4.5	98.2
	200	4.0	103.2
	2000	3.0	106.8
	5000	2.7	104.8
PPc[b]	10	9.1	108.8
	30	5.6	99.2
	400	7.4	99.2
	4000	4.5	96.5
	5000	8.3	101.4
SPEc[c]	10	14.6	110.6
	20	9.2	99.7
	200	6.8	97.6
	2000	5.6	101.0
	5000	4.8	96.7

[a] PP: protein precipitation, no cleavage of drug-ADA bonds (method A). [b] PPc: protein precipitation with prior acid cleavage of drug ADA-bonds (method B). [c] SPEc: solid-phase extraction with prior guanidine cleavage of drug ADA-bonds (method C).

Table 4. Intra-Day Precision and Accuracy of Taspoglutide Quality Control Samples in Rat Plasma (n = 5)

sample preparation	added concentration (pg/mL)	precision (%)	accuracy (%)
PP+SPE[a]	50	12.0	100.7
	100	8.3	90.1
	200	9.0	105.0
	10000	8.7	106.0
	25000	6.2	104.9
PP+SPEc[b]	50	5.4	107.2
	100	8.8	98.6
	2000	7.0	109.5
	10000	11.7	109.7
	25000	9.6	88.7

[a] PP+SPE: protein precipitation followed by solid-phase extraction (method D). [b] PP+SPEc: protein precipitation followed by solid-phase extraction with prior guanidine cleavage of drug ADA-bonds (method E).

Application to Pharmacokinetic Studies. The usefulness of the method was demonstrated through the analysis of more than 900 animal and 700 human study samples so far. Figure 8 shows exemplary concentration-time profiles of taspoglutide in rats. Similar results were obtained from minipig and cynomoglus monkey samples (data not shown). Data from single dose experiments were obtained using the non-dissociating sample preparation (method D) as no ADAs were expected, while after multiple dosing the dissociating assay (method E) was employed. Dog study samples were not available yet. Human plasma samples were measured without ADA-drug cleavage (method A) and after the two dissociating treatments (either formic acid, method B or guanidine, method C). The minor differences in taspoglutide concentrations obtained with and without dissociation could not be correlated to ADA levels. For ADA negative samples, methods A and B performed in the same way: the results obtained with

Table 5. Extraction Recovery and Matrix Effect in Human and Rat Plasma (n = 5)

matrix	sample preparation	added concentration (pg/mL)	matrix factor[d]	relative matrix factor[d]	recovery[e] (%)
human plasma	PP[a]	20	0.77	0.96	101.8
		200	0.85	1.00	90.9
		2000	0.84	1.02	86.8
human plasma	SPEc[c]	20	1.05	1.00	77.8
		200	1.01	0.99	78.3
		2000	1.07	0.99	69.4
human plasma	PPc[b]	30	0.94	1.00	90.3
		400	0.94	0.97	90.1
		4000	0.94	1.03	88.2
rat plasma	SPEc[c]	100	1.13	1.11	85.5
		1000	1.06	0.97	76.2
		10000	1.08	0.92	73.7

[a] PP: protein precipitation, no cleavage of drug-ADA bonds. [b] PPc: protein precipitation with prior cleavage of drug-ADA bonds. [c] SPEc: solid-phase extraction with prior cleavage of drug ADA-bonds. [d] below 1.00: ionization suppression; above 1.00: ionization enhancement. [e] extraction recovery, not interfered by matrix effect.

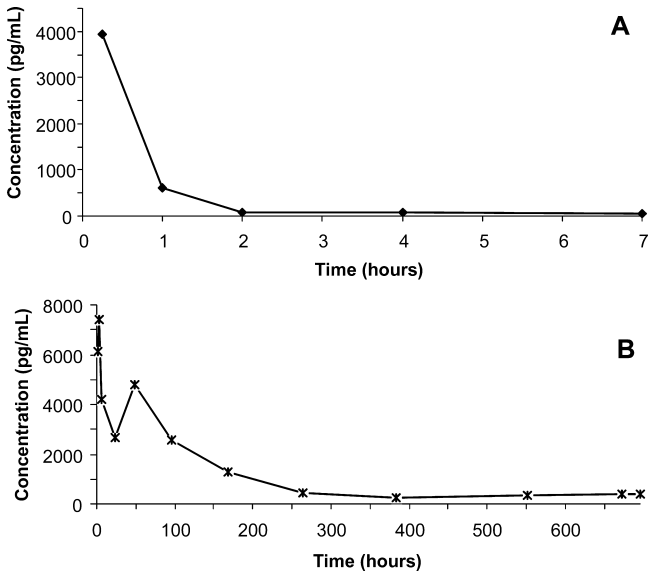

Figure 8. Mean concentration-time profiles of taspoglutide in rats after subcutaneous administration of 1 mg/animal by injection (A) or as slow release formula (B).

the two treatments deviated by less than 20%. Cross-validation of the two ADA-drug dissociating treatments with formic acid and with guanidine resulted in less than 20% deviation from average for 83% of the samples and thus showed that they were equivalent for the investigated samples. Therefore, we will use the more convenient acidic treatment without the need for elaborate SPE in the future.

CONCLUSIONS

This work presents for the first time a highly sensitive and selective LC-ESI-MS/MS method for the determination of the synthetic peptide drug taspoglutide. As the novel orthogonal HILIC to RP column-switching procedure efficiently removed matrix components, simple protein precipitation was sufficient as a sample preparation step for human plasma, also after acidic cleavage of drug-ADA bonds. The cleanup and enrichment of the drug from animal plasma still required off-line SPE which was automated to enable efficient turnaround of high sample numbers. The method performed reliably during all study sample runs: no failed sequences were observed, and the challenging LLOQs of 10.0 and 50.0 pg/mL in human and animal plasma, respectively, were regularly achieved. The low required human plasma volume of only 250 μL compared to 1.5 mL plasma in the previously employed method provided the possibility for repeat analyses, using, that is, dissociating and non-dissociating assay, without the need to increase blood volumes in the clinics. Because of its sensitivity, robustness, throughput capability of two 96-well plates per day and ability to quantitatively dissociate drug-ADA bonds, the method will be employed for analysis of samples from pharmacology and phase III clinical studies.

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SUPPORTING INFORMATION AVAILABLE

Further details on intra-day precision and accuracy of taspoglutide quality control samples in human plasma. This material is available free of charge via the Internet at http://pubs.acs.org.

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