Determining Peptides

A number of methods can be used to apply therapeutic peptides, as very few are non-digestible in the stomach. This begs a number of considerations that need to be made for effective execution

Professor Hermann Mascher at pharm-analyt

Therapeutic peptides are often applied via the following routes: intramuscular (IM), intraperitoneal (IP), intravenous (IV), or inhalative. Only a few peptides are stable in the stomach (acidic plus pepsin digestion) and the intestine (trypsin digestion) after oral application. Determination in plasma is very complex, as far as selectivity is concerned, because millions of endogenous peptides and proteins can be found in plasma.

Usually, the determination of a drug in plasma starts with some questions:

- How much of that drug is applied per kg body weight
- Which route of application is used (IM, IP, IV, oral, or inhalative)
- Is anything known about the first pass effect (leading to metabolites and, therefore, lowering the parent drug concentration)
- Does the molecule show a high volume of bodily distribution (which often means that the molecule shows very lipophilic characteristics or high tissue attraction and, therefore, a fast elimination half-life and low concentration levels in plasma)

A rough estimation of C_{max} values can be established, thus, a rough estimation of the desirable lower limit of quantitation (LLOQ) for a respective assay is possible. The assay's LLOQ should be at least 1-5% of C_{max} values.

With peptides, the questions can be similar if the application route is IM, IP, and especially IV. However, the half-life of a therapeutic peptide is often rather short. Therefore, the assay's LLOQ should be even lower at about 0.1-1% of C_{max} values.

Specific Challenges

Here is where the challenges begin. As many endogenous peptides and proteins in plasma exist, bioanalytical determination has its selectivity limits. Albumin in plasma shows concentration levels of about 5 mg/ml (see Figure 1) and the therapeutic peptide may have a C_{max} value of only about 100 ng/ml. This means that a factor of 50,000 more albumin is present in plasma than C_{max} of the therapeutic peptide and a factor of 5,000,000 more albumin than the LLOQ of the therapeutic peptide. As albumin and other larger proteins can be eliminated by protein precipitation with acetonitrile, many smaller therapeutic peptides (1-5 kDa) stay solubilised in the watery/organic solvent. Thus, centrifugation can eliminate larger proteins as precipitates, however, the next step is more complicated. Peptides are usually amphoteric, meaning they are positively and negatively charged. Therefore, if they are lipophilic (peptides are only seldom rather lipophilic), they cannot be extracted in organic solvents that are not mixable with water, like other lipophilic drugs can be.

Altogether, options to separate a therapeutic peptide from all the endogenous peptides in plasma are limited. Only good separation on a high-performance liquid chromatography column (HPLC) and selective mass spectrometry (MS)/MS detection are the matters of choice. If these attempts are not working appropriately, ion mobility with MS/MS can be used as an alternative tool, and have been for a few years now.

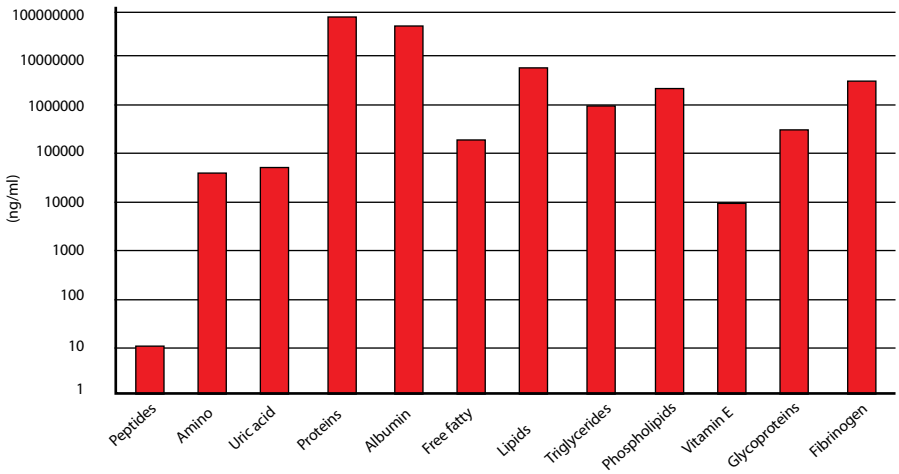

Figure 1: Concentration of selected plasma compounds (logarithmic scale)
Source: Mascher H, HPLC Methods for clinical pharmaceutical analysis: A user's guide, 2012

Waters offer SYNAPT or VION, Sciex offers the tool SelexION. SelexION, on an API65000 QTRAP, can be very exciting in certain cases. Usually, the signal will drop by a factor of approximately five, but the noise, and especially the baseline, will go down more dramatically, which is great for an analyst. Furthermore, a lot of unspecific peaks can be eliminated by using this ion mobility option. As a summary, the signal-

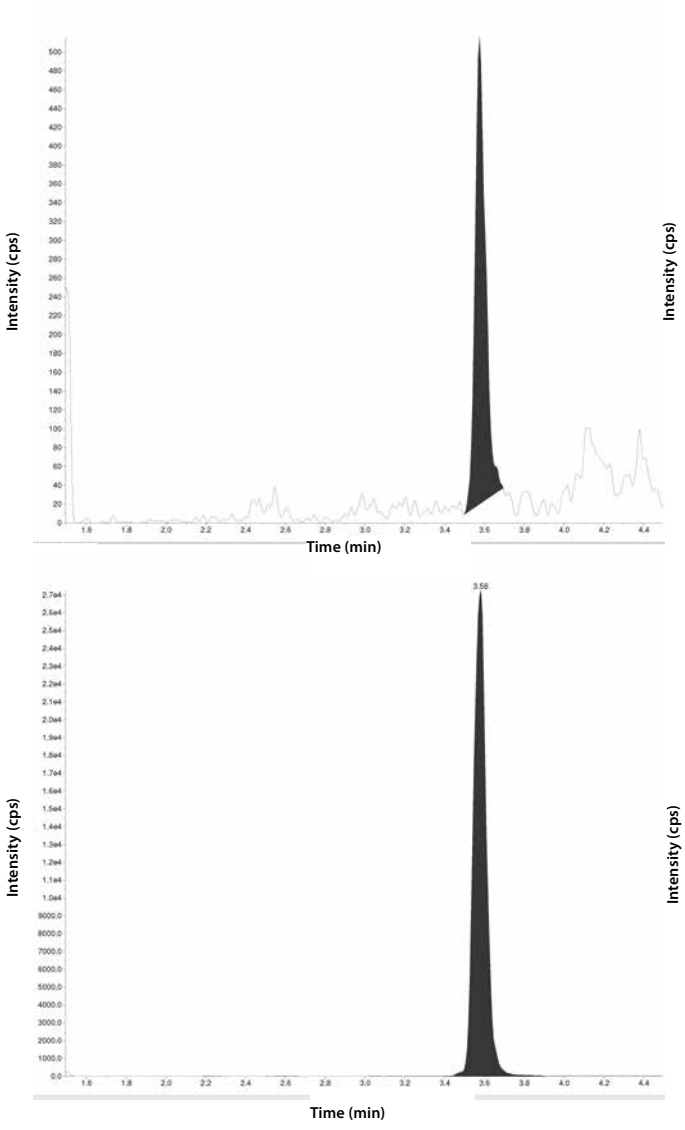

Figure 2: Concentration of 1ng/ml plasma upper chromatography/internal standard lower chromatography
Source: Mascher D et al, Sensitive determination of the peptide AP 301: A motif of TNF-αFrom human plasma using HPLC-MS/MS, J Chromatogr B 908: pp18-22, 2012

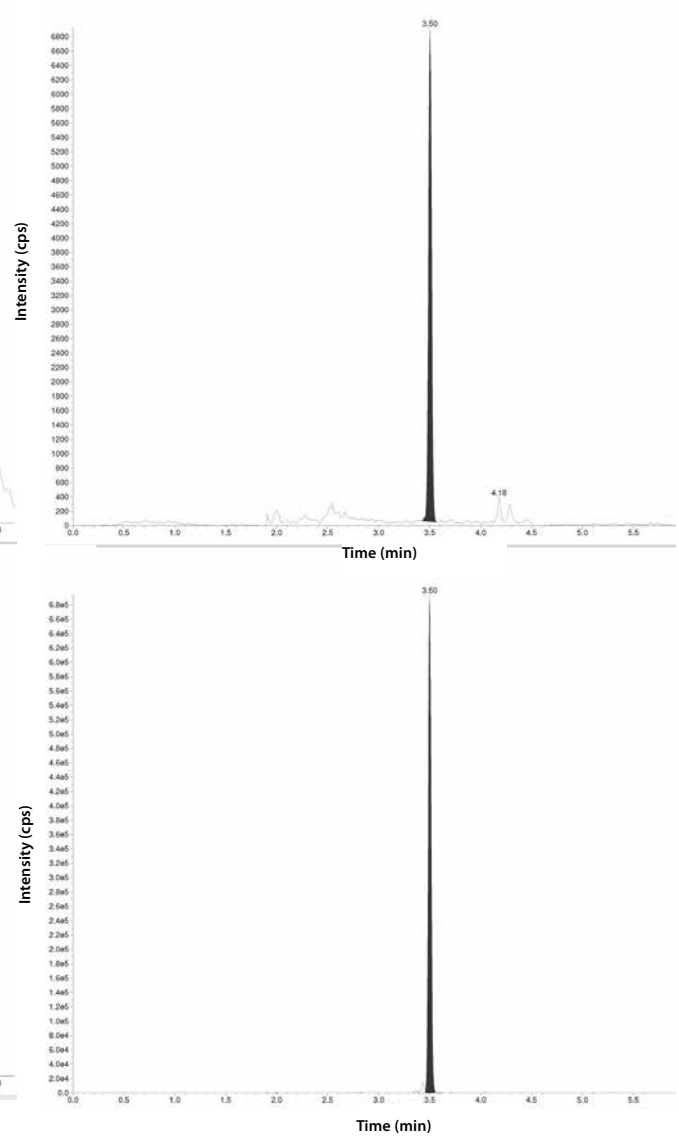

Figure 3: Concentration of 4ng/ml plasma upper chromatography/internal standard lower chromatography

noise ratio often enhances by a factor of five to 10. By using ion mobility in another field of bioanalysis, LLOQs can be lowered significantly for estriol, estrone, and estradiol (especially as estriol and estradiol have a low ion abundance) to 1-5 pg/ml plasma without using the common derivatisation step of the phenolic group, which enhances the ion yield strongly, but delivers a lot of unwanted peaks with similar retention time and multiple reaction monitoring. Besides selectivity problems, the two other main topics are:

Stability issues

As the body is used to 'digest' proteins and peptides, many therapeutic peptides are in danger of being 'digested' by, for example, endogenous proteases. The time after blood drawing (to get plasma and freeze it) must be very short, or a better alternative can be to use chemicals or enzymes (eg, protease inhibitors) to prohibit the destruction of a therapeutic peptide in whole blood or plasma.

Another problem occurs if free SH groups are present in that therapeutic peptide. Often oxidation occurs, so a lot of S-S linkages to many other free thiols (eg, cysteine, glutathion) can be found and almost no therapeutic peptide with the free sulfhydryl (SH) group. Then, the question arises: was it *in vivo* (in the body of an animal or human) oxidation, or did it occur during the process of plasma generation from whole blood or during freeze/thaw processes of plasma? The best way is to add a reductive agent like dithiothreitol (DTT) to plasma behaviour analysis. DTT sets free a lot of S-S linkages. Also, other reducing agents can be used, such as tributylphosphin, though this is very toxic.

Adsorption Effects During Sample Preparation
Basic peptides with (sometimes) some consecutive basic amino acids are in particular danger of being adsorbed on glass walls or even polypropylene walls. Detergents such as Triton X-100 or Tween 80 in low concentrations are helpful for prohibiting such massive concentration versus response effects. Sometimes, similar effects can happen in HPLC-columns, so that the linearity over a factor of 100 or 1,000 is not given. In such cases, similar peptides can be added to the mobile phase or in the injection solution in very low concentration levels to avoid these effects.

Practical Examples

If an inhaled peptide with about 2 kDa (17 amino acids) can be found in the central compartment, does this indicate penetration into blood stream after inhalation of low milligram amounts? Indeed, after method development and Good Laboratory Practice validation in the concentration range of 1-300 ng/ml plasma (by using 50µL of plasma), low concentrations of AP301 can be found with a fast elimination half-life (see Figure 2). This means that this peptide is penetrating the lung wall into the central compartment (blood).

Generally, an isotopically labelled internal standard (preferred C13 or N15, but deuterated also works) is essential for a high quality method. This internal standard can compensate volume variations during sample preparation, but also injection variations and sensitivity issues during electrospray ionisation (preferred for peptides) or atmospheric-pressure chemical ionisation to a large extent. For the described peptide AP301, a 13C-labelled internal standard was used.

Another example is an anticancer peptide with about 700 Da (6 amino acids), which contains a cysteine with a free SH group. In this case, DDT can be used to set free all the bound anticancer peptide molecules in plasma and determine it with a LLOQ of 4ng/ml by using 100µL of plasma (see Figure 3). All in all, determining peptides in plasma is a challenge, but one you might find worthwhile

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Professor Hermann Mascher, bioanalytical consultant and founder of pharm-analyt Labor, has more than 100 publications in peer reviewed journals (with more than a 230 impact factor). Hermann is the inventor of three patents for biomarkers in the area of rare lysosomal storage diseases and has developed analytical methods for dozens of peptides.
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Short communication

Sensitive determination of the peptide AP301 – A motif of TNF-α – From human plasma using HPLC–MS/MS

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ABSTRACT

The AP301 peptide mimics the lectin-like domain of TNF-α. The synthetic peptide AP301 (molecular weight 1923.1 amu) is composed of 17 amino acids and contains an intramolecular disulfide bond between the N-terminal and the C-terminal cysteine. AP301 interacts with the endothelial sodium channel (ENaC) and activates pulmonary liquid clearance both *in vitro* and in animal studies. Currently, AP301 is subject to clinical investigations for the treatment of pulmonary oedema. With HPLC–MS/MS on reversed phase chromatography a determination limit of 1 ng AP301/mL human plasma can be achieved. The MS-ionisation was done with ESI positive. 50 μL of human plasma was mixed with the internal standard (a stable isotope labelled AP301, with a total of 6 carbon 13) in acetonitrile for protein precipitation. After centrifugation a part of the clear supernatant was injected into HPLC–MS/MS. Validation was performed according to FDA-guideline in three batches [U.S. Department of Health and Human Services, Food and Drug Administration (FDA): Guidance for Industry, Bioanalytical Method Validation, May 2001]. By using a 6xC13 isotopically labelled internal standard good precision, accuracy and linearity can be gained. The inter-batch precision (CV) of the quality control samples in human plasma (conc. 2.50/20/240 ng/mL) ranged from 5.54 to 10.15%. The inter-batch accuracy (with reference to the mean value) of the quality control samples in plasma ranged from 96.1% to 99.9%. The analyte was stable in human plasma over three freeze/thaw cycles, or for 4 h at room temperature, or for at least 20 weeks when stored at below −20 °C. This method was used for quantifying AP301 after inhalative application in a phase I-study.

1. Introduction

Determination of peptides of 1–5 kDa from plasma is not easy to do. There are some hindrances: First, there are lot of endogenous substances which can disturb a specific determination. In Fig. 1 [2] some possibly disturbing endogenous substances can be seen, often with much higher concentration (see log concentration axes). Second, there are many similar peptides in plasma, sometimes in concentration ranges up to 1 μg/mL. As peptides are usually hydrophilic and amphoteric specific sample clean up is almost impossible. Third, a lot of peptides are destroyed within minutes by proteinases in plasma, therefore stability in the gained plasma could be very problematic [3]. Forth, some peptides are strongly bound to usually large proteins therefore extraction respectively recovery could be quite a problem [4].

The Tumour Necrosis Factor alpha (TNF-α) is a cytokine composed of three polypeptides, each containing a binding domain to TNF-receptors and a lectine-like domain located at the "tip" of the molecule. However, due to its pro-inflammatory signalling properties the clinical use of TNF-α is limited. The AP301 is a fully synthetic peptide whose molecular structure is based on the lectin-like domain of TNF-α. By contrast to TNF-α, AP301 does not contain the TNF-receptor binding domain. AP301 is designed to activate the pulmonary epithelial sodium channel (ENaC) *via* binding to the extracellular carbohydrate moiety of ENaC. Activation of sodium ion transport by ENaC results in an accelerated oedema clearance in the airspace *in vitro* and *in vivo*. AP301 is being developed for the treatment of oedematous respiratory failure in life-threatening conditions such as Acute Lung Injury, Acute Respiratory Distress Syndrome and lung transplantation.

AP301 (amino acid sequence CGQRETPEGAEAKPWYC) contains an intramolecular disulfide bond between the N-terminal and the C-terminal cysteine. AP301 is water soluble and can be administered into the lung by oral inhalation as aerosol.

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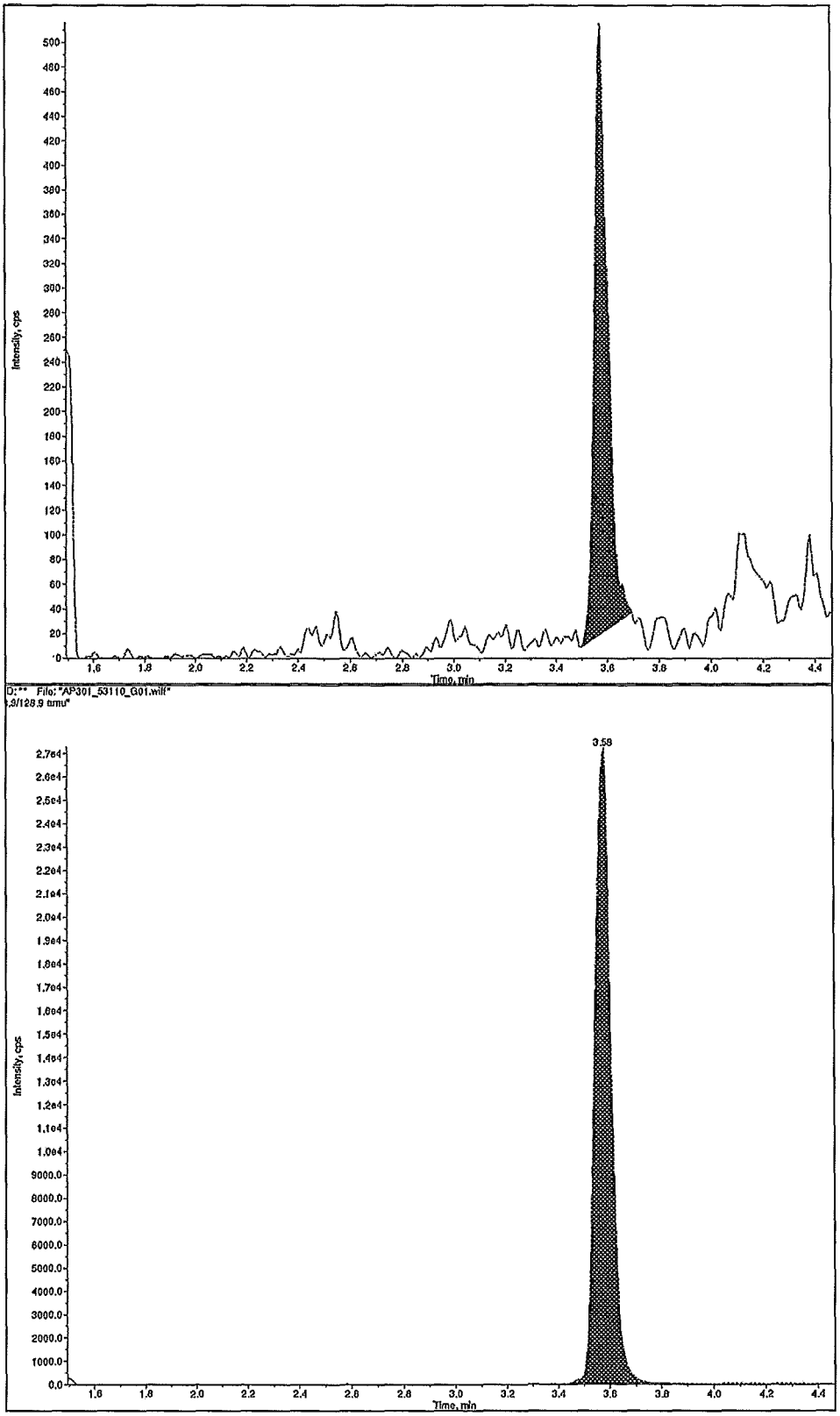

Fig. 2. Chromatograms of plasma Std. 1 at 1.00 ng/mL, upper trace AP301 (642–129 *m/z*), lower trace IS (644–129 *m/z*).

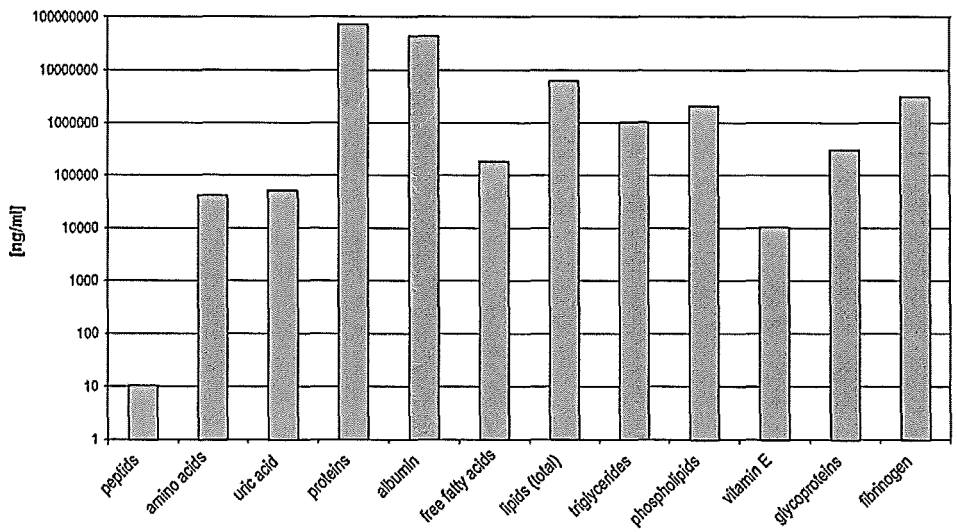

Fig. 1. Concentration of selected plasma compounds (logarithmic scale).

Currently, AP301 is subject to a phase I clinical study for safety and tolerability in healthy human volunteers.

2. Materials and methods

2.1. Instrumentation

The MS/MS system used for these experiments was an API 5000 (PE Sciex, Canada) in combination with the positiveTurbo V-Ionspray (ESI). Gas for the MS was delivered from a nitrogen generator (Whatman, USA). The auto sampler was a series 200 auto sampler from Perkin Elmer (Germany) and the liquid chromatography (LC) system consisted of a HP 1100 pump (Agilent Technologies, USA). The column oven was a HP 1100 column oven (Agilent Technologies, USA). The data system consisted of a PC based on Windows NT 4.0 (SP 5) with Analyst 1.4.2 software. The analytical column was a YMC Pack Pro C8, 100 mm × 3 mm, 3 μm (YMC, Japan).

2.2. Chemicals

All chemicals used in this study were of analytical reagent grade.

AP301 was provided by SENN Chemicals (Switzerland). AP301 [13C6] was from piCHEM (Graz, Austria). Human Plasma was provided by "Blutplasmazentrum Koblenz" in Germany.

2.3. Preparation of calibration standards, quality control samples and other control samples

To calibrate each batch, calibration standards were made at several concentration levels. The highest calibration level was prepared by adding a defined volume of 20% methanolic analyte solution to human plasma. All other calibration standards were prepared by a more highly concentrated calibration standard to blank human plasma samples. Calibration standards were spiked at: 1.00, 2.00, 5.00, 10.0, 30.0, 90.0, 150, and 300 ng/mL of human plasma. Quality control samples were spiked at 2.50, 20.0, and 240 ng/mL of human plasma and LLOQ samples were spiked at 1.00 ng/mL.

2.4. Sample preparation

All samples used within a validation sequence were prepared for analysis as follows:

50 μL of sample was transferred into a glass vial. After 100 μL of the internal standard (IS) working solution (AP301 [13C6] at 67.6 ng/mL; in 80% acetonitrile; same working solution used for all samples of a batch) had been added, samples were shaken vigorously for about 2 min.

After 15 min storage at 2–8 °C, samples were centrifuged at 4000 rpm for 3 min. Aliquots of approx. 150 μL were transferred into conical autosampler vials. The organic part of the sample was evaporated in a Rotation Vacuum Centrifuge (15 min, 10 min thereof at 45 °C, pressure 100 mbar, security pressure 110 mbar). Finally, the samples were sealed with an aluminium crimp cap and injected into the HPLC–MS/MS system within 45 h, or stored at below −20 °C prior to analysis.

2.5. Chromatographic conditions

The mobile phase 'A' used was 1.0% formic acid in water *vs.* the mobile phase 'B' which was 1.0% formic acid in methanol. The gradient was isocratic at 10% B for 0.5 min, then from 10% B to 44% B in 3.4 min, then isocratic at 80% B for 1.0 min, and a re-equilibration at 10% B for 2.1 min. The column used was a YMC Pack Pro C8, 100 mm × 3 mm, 3 μm (YMC, Japan) at 40 °C. The flow rate was 0.80 mL/min, and the injection volume used was 15 μL. Approximate retention times were 3.6 min for AP301 and its internal standard.

2.6. Mass spectrometric conditions

An ESI source was used in positive ion mode at 5.5 kV on an API 5000. The vaporizer temperature was set at 700 °C, gas 1 at 70 psi, gas 2 at 60 psi and the curtain gas at 40 psi. MRM transitions were 642 $[M+3H]^{3+}$ to 129 *m/z* for AP301 and 644 $[M+3H]^{3+}$ to 129 *m/z* for AP301 [13C6] (internal standard).

2.7. Method validation

The analytical method was validated in three batches [1].

2.8. Method linearity

The calibration range was from 1.00 to 300 ng/mL AP301 in human plasma.

Table 1
Inter-batch data of the quality control samples in human plasma.

Sequence	Low-range (exp. conc. 2.50 ng/mL)			Mid-range (exp. conc. 20.0 ng/mL)			High-range (exp. conc. 240 ng/mL)		
	Mean [ng/mL]	CV [%]	Accuracy [%]	Mean [ng/mL]	CV [%]	Accuracy [%]	Mean [ng/mL]	CV [%]	Accuracy [%]
Batch 1	2.50	11.20	100.2	18.1	13.26	90.6	242	5.63	100.9
Batch 2	2.56	4.67	102.5	19.7	10.30	98.4	225	4.26	93.8
Batch 3	2.42	4.47	96.9	19.8	5.43	99.2	231	4.77	96.3

2.9. Precision and accuracy

Five replicates of quality control samples were each analysed at three concentration levels.

2.10. Specificity

At least six specificity samples (plasma of six different volunteers) were analysed with and without internal standards (IS) in the same validation batch.

2.11. Stability

AP301 stability in human plasma was tested for four hours at room temperature (about 25 °C), and three freeze/thaw cycles in plasma were performed. Also LTS (long term stability) over 20 weeks at below −20 °C was performed.

2.12. Recovery

The recovery of the analyte was calculated at each QC-level.

3. Results and discussion

3.1. Method development

It is always critical to determine peptides in human plasma. Three types of problems can occur. First, a lot of other endogenous peptides which could have the same molecular weight at sometimes much higher concentration than the analyte. Second, co-precipitation of the analyte with the huge amount of proteins by using an organic solvent like acetonitrile. Third, the analyte may be sensitive to proteases or similar enzymes in plasma and therefore stability in plasma – also for a short time – may not be given.

For AP301 point 2 and 3 did not make any problem. Point 1 – unselectivity – could be solved through good HPLC separation and MRM transitions combined with specific ionisation conditions (evaporation temperature and voltage).

3.2. Precision, accuracy and linearity

The calibration curve was linear in the range from 1.00 to 300 ng/mL AP301 in human plasma (Fig. 2: Chromatograms of plasma Std. 1 at 1.00 ng/mL). The respective inter-batch data are presented in Table 1. LLOQ summary data are presented in Table 2.

3.3. Specificity

The specificity of the method was determined by screening six samples of both blank human plasma. The specificity criteria were fully met for the analyte. This was also used for checking the matrix effect.

3.4. Stability

AP301 is stable in human plasma for at least 20 weeks at below −20 °C. Stability of the analyte in plasma is at least four hours at room temperature (about 25 °C). Also three freeze/thaw cycles in plasma can be performed.

3.5. Recovery

Recovery was found to be 70.5% for AP301 and 80.5% for its internal standard.

3.6. Results after application in humans

Based on Ethic Committee and Medicines Agency approval, the safety, tolerability and pharmacokinetic profile of orally inhaled AP301 was assessed in a phase I clinical study in 48 healthy male volunteers. AP301 was orally inhaled at 6 planned doses between 0.07 mg/kg to 2 mg/kg AP301.

Lung function parameters, such as FEV1 and PEF, were not affected by AP301. Physical examinations resulted in no signs of spastic cough and severe xerostomia. Exhaled nitric oxide did not increase. Vital signs, ECG and safety laboratory parameters were unaffected by AP301. In regards to bioanalysis very low levels of

Table 2
LLOQ summary data.

Sequence	Calculated concentration [ng/mL]						Mean [ng/mL]	CV [%]	Accuracy [%]
	Value 1	Value 2	Value 3	Value 4	Value 5	Value 6			
LLOQ-1 (expected concentration 1.00 ng/mL)									
Batch 1	1.27	1.11	1.01	1.06	1.01	1.08	1.09	8.81	108.9
Batch 2	1.19	1.13	1.27	1.23	1.17	1.14	1.19	4.57	119.0
Batch 3	1.04	0.936	0.796	1.12	0.966	0.856	0.952	12.37	95.2

Acceptable range for mean (80–120%): 0.801–1.20 ng/mL

Inter-batch precision and accuracy (reproducibility), n = 3 batches	
Mean	1.08 ng/mL
CV	12.37%
Number	18
Exp. conc.	1.00 ng/mL
Accuracy	107.7%

AP301 can be found only after the highest inhaled dosages. These levels were at 1–5 ng/mL human plasma and can be detected within the first hour after inhalation only.

4. Conclusion

The quantitative determination of AP301 – the lectin-like domain of TNF-α – is described here using 50 μL human plasma only down to an LLOQ of 1 ng/mL.

This HPLC–MS/MS-method was applied to samples of a phase I clinical study in 48 healthy male volunteers.

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