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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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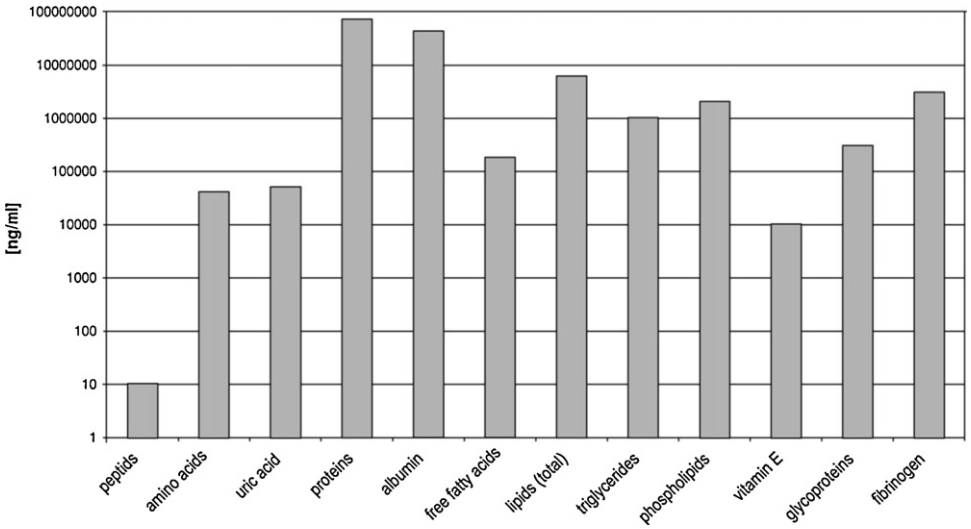

Fig. 1. Concentration of selected plasma compounds (logarithmic scale).

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.

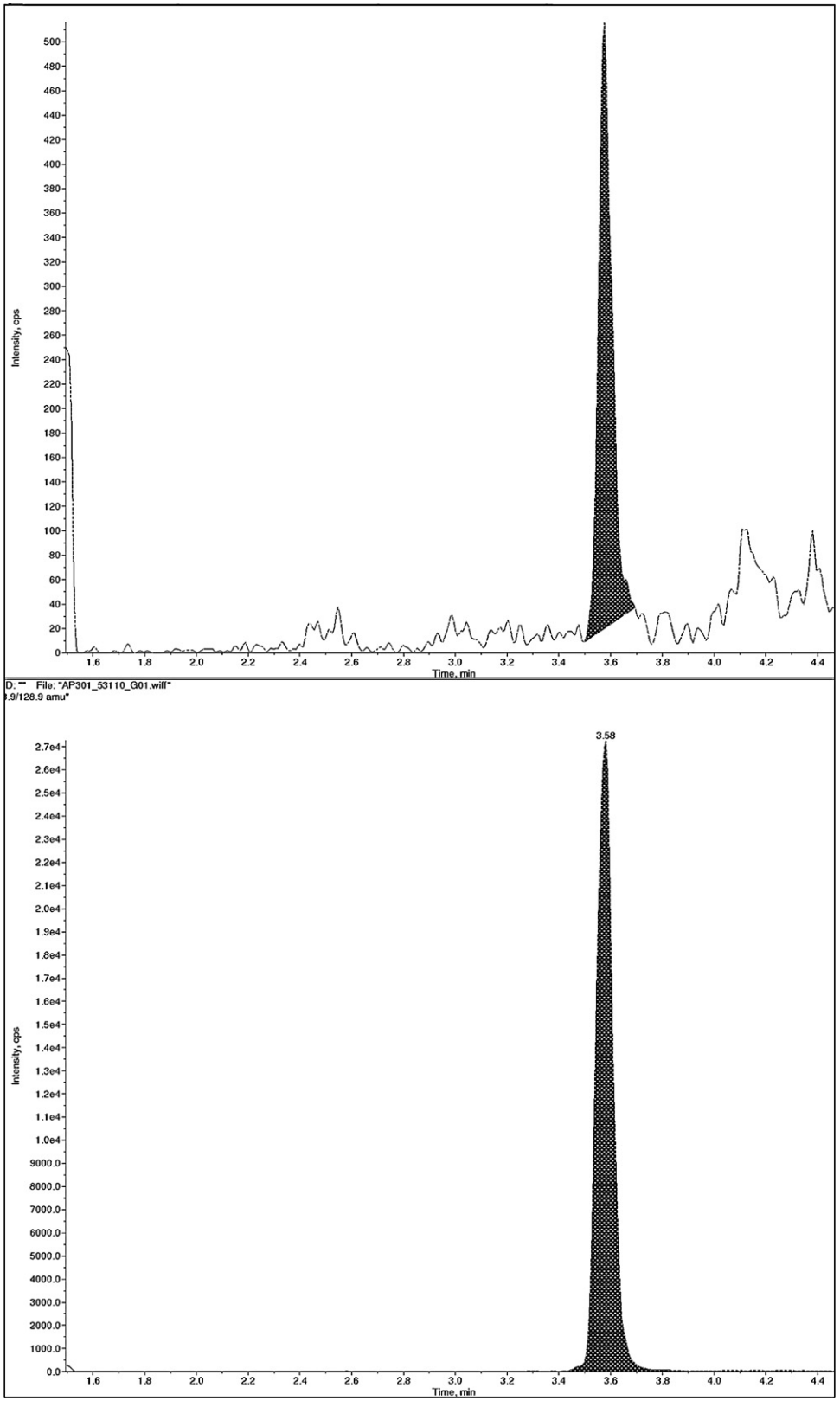

Fig. 2. Chromatograms of plasma Std. 1 at 1.00 ng/mL, upper trace AP301 (642–129 *m*/*z*), lower trace IS (644–129 *m*/*z*).

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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