This is a pre- or post-print of an article published in
Gross, P.C., Burkart, S.C., Müller, R.
Analytics of the therapeutic peptide aviptadil by sheathless CE-MS and comparison with nanoRP-HPLC-MS
(2014) Journal of Pharmaceutical and Biomedical Analysis, 88, pp. 477-482.

Analytics of the Therapeutic Peptide Aviptadil by Sheathless CE-MS and comparison with nanoRP-HPLC-MS

PETER C. GROSS[1,4], SONJA C. BURKART[1,3,4], ROLF MÜLLER[1,2,3]*

[1] Biotech Processes and Analytics Department, PharmBioTec GmbH, D-66123 Saarbrücken, Germany

[2] Helmholtz Institute for Pharmaceutical Research Saarland, D-66123 Saarbrücken, Germany;

Helmholtz Centre for Infection Research, Saarland University, D-66123 Saarbrücken, Germany

[3] Department of Pharmaceutical Biotechnology, Saarland University, D-66123 Saarbrücken, Germany

[4] These authors contributed equally to this work.

* Helmholtz-Institute for Pharmaceutical Research Saarland; Helmholtz Centre for Infection Research and Pharmaceutical Biotechnology; Saarland University; Campus, Building C2 3; 66123 Saarbrücken; Germany
Phone: +49 681-302-70200. Fax: +49 681-302-70202. E-Mail: rom@mx.uni-saarland.de

Author's E-mail addresses: rom@mx.uni-saarland.de (Rolf Müller); p.gross@pharmbiotec.de (Peter C. Gross); s.burkart@pharmbiotec.de (Sonja C. Burkart)

ABSTRACT

Purification and quality control of therapeutic peptides is often performed by one single method, RP-HPLC. As usage of an orthogonal technique is highly advisable for quality assurance, capillary electrophoresis (CE) employing a coated capillary coupled via a sheathless interface to a mass spectrometer was applied in parallel. The basic therapeutic peptide aviptadil served as a model substance to study the impurity profiles revealing 15 detectable impurities using CE-MS, two were detected by an appropriate nanoRP-HPLC-MS method. None of the impurities detected by CE were observed in LC and vice versa. The LOD in CE-MS was determined in the base peak electropherogram at ~1 fmol, a value 2500 times smaller than the LOD found in nanoRP-HPLC-MS (3 pmol). In nanoRP-HPLC-MS only 0.2 % of the extrapolated CE-MS signal for a 25 ng aviptadil load was observed. We conclude that both, the LOD as well as the impurity profile of aviptadil, as analyzed by nanoRP-HPLC are influenced by both, the ligand-derivatized silica matrix and the flow-rate. Peptides may disappear completely and their variable emergence may lead to the determination of incorrect ratios as present in the sample.

Keywords: sheathless CE-MS, therapeutic peptides, unspecific peptide adsorption, nanoLC-MS, impurity analysis, quality control

1. Introduction

Peptides are continuously attracting attention as therapeutics, e.g. insulines, exenatide, lixisenatide, liraglutide, just to name a few. From a manufacturing perspective solid phase peptide synthesis (SPPS) is the preferred way of production, which is also reflected by the fact that most of the recent blockbuster peptides are produced as such.[1] However, because of the imperfect coupling efficiency on each step of a multistage synthesis, truncated and also substituted peptide variants of the target peptide are common impurities in chemically produced peptides. Only the resolution provided by Reversed-Phase-High-Performance-Liquid-Chromatography (RP-HPLC) in preparative dimensions allowed to take advantage of the fast SPPS method for the production and purification of peptide products at accepted purity.[2] At the same time, RP-HPLC, in its different variations, is also the method of choice for the quality control of chemically synthesized peptides. However, main peak heterogeneities observed in preparative RP-HPLC might be still present in analytical RP-HPLC and quantitation of the overlapping species could be difficult. Consequently, an enormous effort for method development could be necessary to develop highly selective RP-HPLC methods to characterize the final peptide drug comprehensively.

A method based on a different physical separation principle compared to RP-HPLC, such as capillary zone electrophoresis (CZE), is in theory ideally suited to analyze chemically synthesized peptide drugs purified by RP-HPLC for the presence of impurities. However, CZE suffers from being less sensitive in case of UV detection as compared to RP-HPLC, therefore demanding stacking techniques to apply higher sample loads could be necessary.[3] One alternative, the online coupling of CE to MS detectors with electrospray ionization, is not as straightforward and widely used as in case of RP-HPLC, but bears the potential for easier substance identification and to overcome the sensitivity problem. Here, two electric circuits, one from the CE and another one from the ESI interface have to coincide at the tip of the CE separation column. Several reviews are available summarizing the current state of the art.[4-7] To the best of our knowledge in the field of CE-MS only sheathflow systems are commercially available. Sheathflow systems provide an opportunity to add a carrier flow to the CE eluate to establish stable ESI spray conditions in a variable manner depending on the actual method and/or analyte. On the other hand sheathflow systems are working with flow rates at least one order of magnitude higher than the intrinsic CE flow rates and by that the sensitivity is decreased in cause of the less efficient ESI ionization process and due to sample dilution.[4] In 2007, Moini presented a new CE-MS interface based on a porous fused silica tip, which is produced by etching the CE capillary tip with hydrofluoric acid.[8] It was found by Busnel et al. (2010), that a prototype of Beckman Coulter based on this interface is capable of generating a stable spray with flow-rates ranging from less than 10 nL/min up to 340 nL/min with concentration limits of detection in the subnanomolar range.[9] Haselberg et al. (2010) applied this new type of sprayer to the analysis of four intact model proteins up to a size of ~30 kDa.[10] Furthermore they compared a sheath-liquid CE-MS interface with the sheathless system and described a 50-140 fold improved detection limit for the latter system using the same capillary.[10] The suitability of the prototype for peptide analysis was further evaluated by Faserl et al. (2011).[11] The authors have shown that less than 30 amol of the small neutral peptide angiotensin I were required for detection in the base peak electropherogram. By comparison with LC-MS, they have shown, that low molecular mass peptides (below 1400 Da) were preferentially identified by CE-MS.[11] Recently, Heemskerk et al. applied this new CE sprayer architecture successfully to the glycopeptide analysis of antibodies together with a transient isotachophoresis. They have shown, that the method provides information on IgG Fc glycosylation, even for those samples with IgG1 concentrations below the LODs of conventional methods like nanoRP-HPLC-MS.[12]

We were interested in alternative methods besides RP-HPLC for the quality control of chemically synthesized therapeutic peptides purified by preparative RP-HPLC. Therefore, on the basis of a CE capillary prototype provided by Beckman Coulter and constructed according to the Moini sprayer, a CE-MS method for the chemically synthesized peptide therapeutic aviptadil (vasoactive intestinal peptide, VIP) was developed. To study the differences in selectivity and sensitivity in an exemplary (not fully exhaustive) manner between CE-MS and RP-HPLC-MS, a nanoRP-HPLC-MS method was also applied for aviptadil. The GMP produced basic therapeutic peptide aviptadil (28 amino acids; MW 3,324.8 Da; pI 9.8) is used in clinical trials and applied as inhalative therapeutic in cases of pulmonary hypertension.[13] Abbaye et al. investigated the pre-electrospray ionization factors, e.g. by observing the number of multiple protonations and ESI response for VIP[14]. In the work presented here we focused on the potential of the sheathless CE-MS separation technique for the field of pharmaceutical peptide drug quality control in terms of selectivity, sensitivity and quantification.

2. Experimental Section

2.1 Materials. Fully synthetic GMP produced aviptadil was obtained from Bachem (Bubendorf, Switzerland, purity >99 % according to Bachem's RP-HPLC analysis) as a lyophilisate and was already stored for 1.5 years at -80 °C at the beginning of the study. Formic acid (50 %), ammonium formate (99%) and acetonitrile of analytical/HPLC-MS grade were obtained from Sigma-Aldrich (Schnelldorf, Germany). Anhydrous methanol (99.8 %) was received from Acros Organics (Geel, Belgium). The positively charged coating reagent trimethoxysilylpropyl modified polyethyleneimine (PEI) was obtained from ABCR (AB127731 Karlsruhe, Germany) and ESI-L Low Concentration Tuning Mix from Agilent (Waldbronn, Germany). Ultrapure water was prepared by a Millipore Milli-Q Integral 10 water purification system (Schwalbach, Germany).

2.2 Capillary electrophoresis. A Beckman Coulter PA800 Plus system (Brea, CA, USA) was coupled to a Bruker maXis 3G mass spectrometer (Bremen, Germany) via a sheathless porous capillary prototype (total length 100 cm; i.d. 30 µm; o.d. 150 µm; total capillary volume 707 nL), provided and manufactured by Beckman Coulter. The sprayer was fixed in an x-y-z-stage and the tip was 1.5-3.0 mm positioned in front of the ESI inlet. The last ~5 cm of the CE tip are surrounded by a stainless steel needle, filled with a conductive liquid, which was grounded to close the electrical circuit of CE, as well as ESI. A detailed description of the Beckman Coulter sheathless prototype is given elsewhere.[10; 11] As background electrolyte (BGE) and conductive liquid solely 50 mM formic acid pH 2.9 (pH adjusted with 50 mM ammonium formate) was used, which was prepared by diluting 1.89 mL of 50 % formic acid to 500 mL with ultrapure water. Aviptadil lyophilisate was weighed on a micro scale (Mettler Toledo XP56, Giessen, Germany) and dissolved in ultrapure water to receive a stock solution of 100 µg/mL. All other concentrations were obtained by dilution of the stock solution with ultrapure water. The clear solutions have been centrifuged prior to injection for 10 minutes at 15,000 g to avoid capillary blocking by sub-visible particles.

Capillary electrophoresis conditions were as follows: The separation procedure comprised a first rinsing step of the separation capillary with BGE (50 psi; 9 min) and a rinsing of the secondary capillary at the outlet-site with BGE (50 psi; 1 min). The sample was hydrodynamically injected for 10 s at 5 psi (calculated injection volume 7.7 nL; ~1.1 % of the capillary volume) followed by a plug of BGE (5 s at 2 psi). The separation was performed at a temperature of 25 °C and a voltage of 30 kV with reversed polarity and a conductive liquid rinse with 0.5 psi. Maximum separation time was 30 min.

2.3 Capillary Coating Procedure. The Beckman Coulter sheathless prototype capillaries were coated with the positively charged PEI according to the protocol described in U.S. Patent 6,923,895

B2. Shortly, the capillary was rinsed with the coating solution (250 µL PEI diluted in 1 mL of anhydrous methanol) and incubated overnight, afterwards flushed with air and anhydrous methanol.

To store the coated capillary overnight it was rinsed with water-free methanol (50 psi; 10 min), water (50 psi; 5 min) and air (50 psi; 5 min). This basic coating causes an inverse EOF, which was determined with the help of pure water as EOF marker [11] to be between 80 nL/min-130 nL/min.

2.4 Mass Spectrometry. Electrospray Ultra-High Resolution tandem TOF (UHR-Qq-TOF) mass spectrometer maXis 3G from Bruker Daltonics (Bremen, Germany) was used throughout the study. The mass spectrometer was daily calibrated with Agilent ESI-L Low Concentration Tuning Mix via direct-infusion by a syringe pump prior to the analysis. Full scan MS spectra were acquired from m/z 150 to 2000 in positive ion mode with a scan rate of 5000/s and a spectra summation factor of 2500 scans. The capillary voltage was set to -1.4 kV, the dry gas was set to a flow rate of 4.0 L/min and a temperature of 180 °C. The fragmentation analysis was performed during a standard separation run with 100 µg/mL aviptadil. The conditions of the isCID-MS/MS fragmentation process have been optimized prior to the analysis by direct infusion. The isCID energy was set to 90.0 eV in the low-pressure region and 10 eV for the collision cell. The CE-/nanoLC-MS data were evaluated with the Bruker DataAnalysis software.

2.5 nanoLC-MS. Aviptadil was analyzed with a Bruker-Proxeon EASY-nLC II nanoLC system (Bremen, Germany) on a Thermo-Fisher EASY C18-A2 column (PN SC200, Dreieich Germany) with a total length of 10 cm an i.d. of 75 µm and 3 µm end-capped silica particles (Dr. Maisch ReproSil-Pur C18-AQ particles; 120 nm pore size). The system was coupled to the maXis Q-ToF mass spectrometer via the Bruker ESI nano sprayer interface (PN 255780). The gradient (solvent A: 0.1 % formic acid in water; solvent B: 0.1 % formic acid in 50 % acetonitrile) started after 1 min of equilibration with 100 % of solvent A and risen up linearly to 100 % B during 15 min. The flow rate was set to 500 nL/min and the injection volume was kept constant at 1 µL for all samples. The settings of the maXis QToF were -4.5 kV for the capillary voltage, -500 V for the end plate offset and dry gas flow was 4.0 L/min at 180 °C.

3. Results and discussion

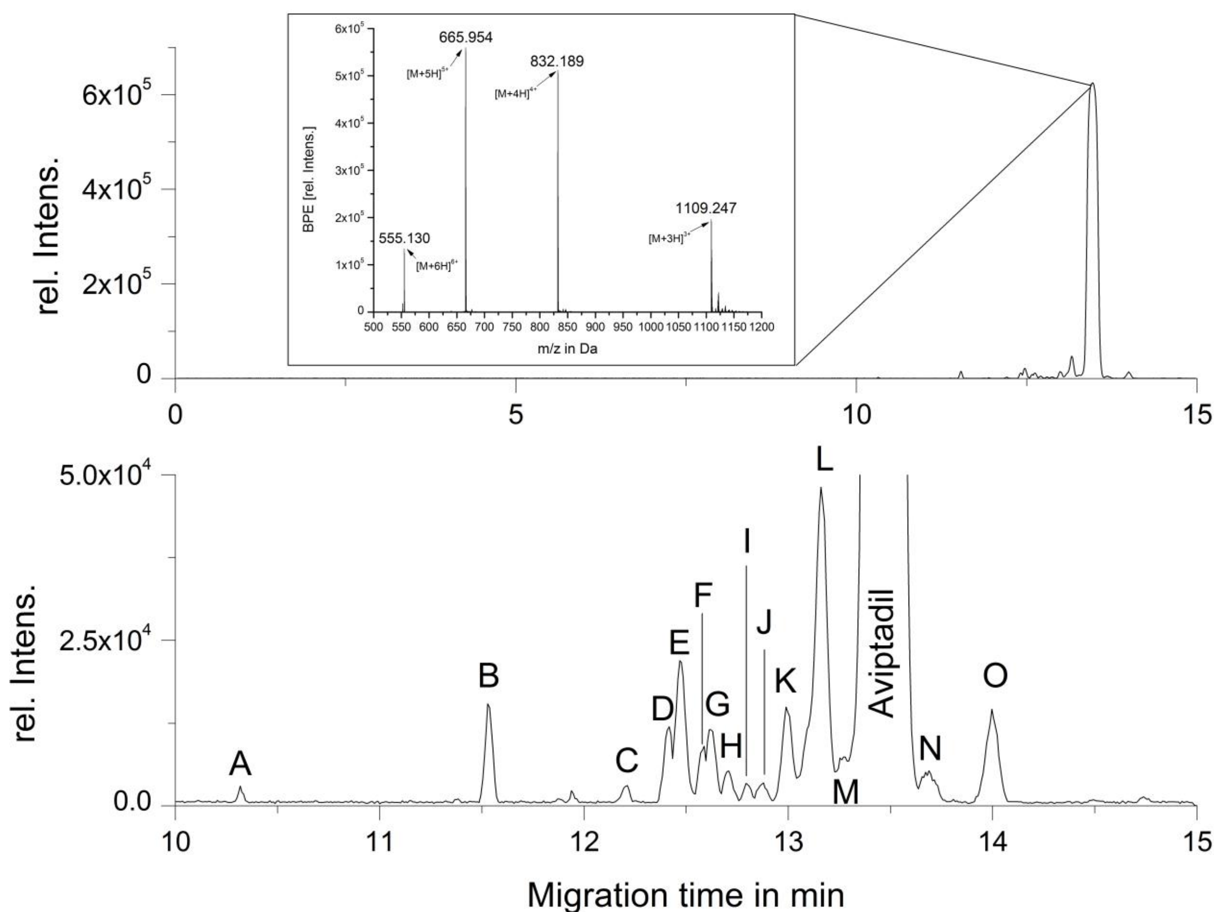
In CZE, analytes are separated according to their different specific electrophoretic mobilities µ, which are defined as the proportionality constant between the analyte migration velocity and the electric field strength[15]. Assuming Stokes' friction, µ is directly proportional to the charge of the analyte and indirectly proportional to its molecule radius.[15] In RP-HPLC the separation principle is completely different and relies mainly on the hydrophobic properties of the molecule's surface. These differences were our most important motivation to establish CE-MS as an orthogonal separation technique in comparison to LC-MS.

First trials using uncoated capillaries for CZE analysis were not successful, probably due to the highly basic nature of aviptadil. After several experiments with different dynamic and static coating agents, the static coating based on the basic polymer polyethyleneimine (PEI) was selected for further experiments. This basic coating especially suppresses the adsorption of basic peptides and leads to a constant and strong (inverse) electroosmotic flow (EOF), which is a prerequisite for working with a sheathless CE-MS interface. Furthermore, this coating is known to be robust and stable over long time-periods.

3.1 Specificity

The sequence of aviptadil is HSDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2, overall it possesses 28 amino acid residues and has a monoisotopic mass of 3,323.75 Da. In Figure 1, the base-

180 peak-electropherogram of a solution containing 100 µg/mL aviptadil is depicted, the insert graph
181 shows the measured mass spectrum for the main peak at 13.5 min. Four different charged species are
182 observed with z-values ranging from 6 to 3. The monoisotopic mass for the main peak at 13.5 min is
183 calculated by charge deconvolution to 3,323.73 Da, which differs from the calculated value only by
184 0.02 Da. A more detailed analysis of the mass spectrum in Figure 1 revealed some additional masses
185 (not shown), which were interpreted after charge deconvolution as aviptadil missing a methionine (-
186 131.06 Da), loss of an ammonium ion (-17.02 Da), a methionine oxidation (+15.99 Da), a sodium
187 adduct (+21.98 Da), a di-water adduct (+35.96 Da) and a tert-butyl-VIP derivative (+56.00 Da).
188



189

190 **Figure 1.** Base-Peak-Electropherogram of 100 µg/mL aviptadil solution in water, separated on a PEI coated capillary.
191 Upper: Complete electropherogram with the mass spectrum of the main peak as insert. Lower: Enlarged view of the same
192 electropherogram with annotation of the observed impurities above the ICH reporting limit of 0.05 Area%. In Table 1 a
193 peak/mass-table is given together with a partial interpretation of the peaks.

194

195 The main peak at 13.5 min in Figure 1 was further analyzed by isCID-MS/MS (in-source-CID-
196 MS/MS) in view of sequence confirmation. Here, a high-voltage induced collision in the medium
197 pressure region of the MS instrument is combined with a second CID in the MS collision cell. Both
198 CID's have been performed without the selection of a predefined precursor ion for technical reasons.
199 Generally, for this approach a high peak homogeneity is mandatory, which is achieved by the high-
200 resolving power of the CZE method in front of the ESI inlet. We were obliged to choose this
201 technique mainly because of the limited fragmentation pattern observed during conventional CID
202 MS/MS experiments. The resulting isCID-MS/MS spectrum after both fragmentation steps is

depicted in Figure 2 with interpretation. Fifteen out of the theoretically expected 27 b-ions of aviptadil could be detected in one or two charge states. The intensities of the found b-ions have a minimal signal-to-noise ratio of 6:1 and allow a sequence confirmation for the amino acids from 1 to 6 and from 20 to 28, resulting in a sequence coverage of 54 %. Further improvement was not achieved. The amount of detected y-ions is limited to the six N-terminal ions.

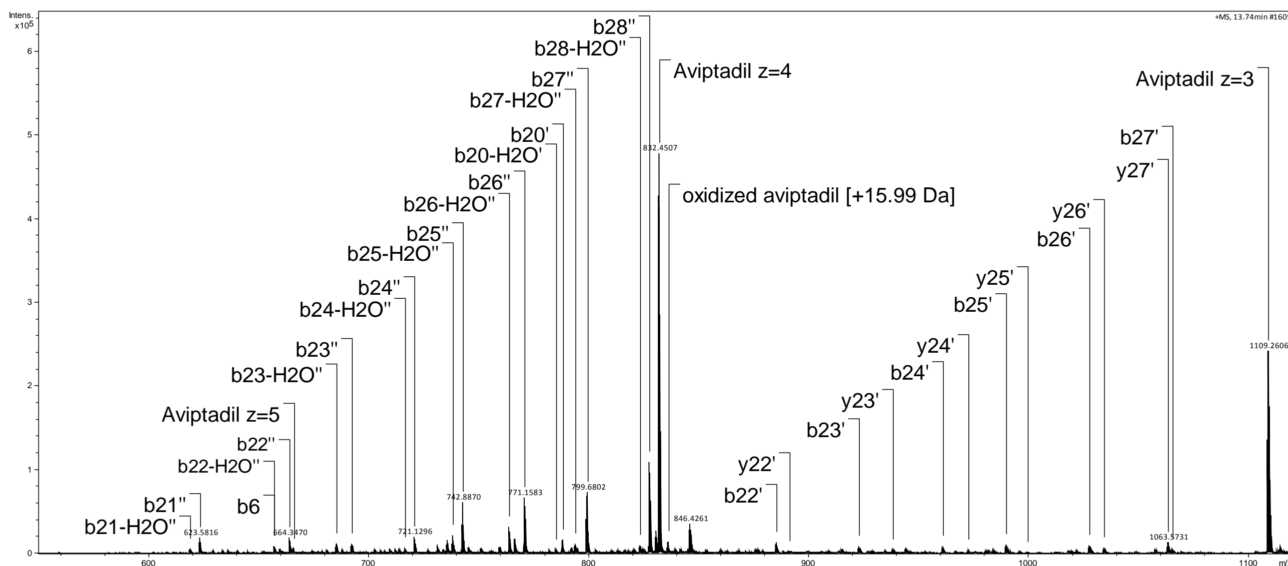


Figure 2. Fragmentation mass spectrum of aviptadil main peak after both fragmentation steps (isCID as well as collision cell induced CID) including all assigned b- and y-ions between 550 m/z and 1150 m/z. B-ions marked without apostrophe are singly charged, b-ions with one apostrophe are triply charged and with two apostrophes are quadruply charged.

3.2 Selectivity / Detection of Impurities

In addition to the aviptadil main peak shown in Figure 1, 15 impurities or drug related substances above 0.05 Area% were observed in CE-MS and are summarized in Table 1. The overall purity determined with the CE-MS method for the 1.5-years stored GMP sample by this analysis was 91.0 %. The same sample was also analyzed by nanoRP-HPLC-MS with the identical mass spectrometer as detector, resulting in estimation of the overall purity to 89.2 %. Even if the determined overall purity by CE-MS and nanoLC-MS was found in the same range, the detected impurities are completely different. In our nanoLC-MS method only two additional peaks were observed, one of them could be interpreted as VIP oxidized at the methionine residue (3339.76 Da, Δ+16.03 Da), while the second was unidentified. The complementary characteristic of both methods is obvious. We observed two fundamentally different impurity profiles; CE resolves more impurities, but the important methionine-oxidized species completely resolved in nanoRP-HPLC, co-migrates with the main peak in CE.

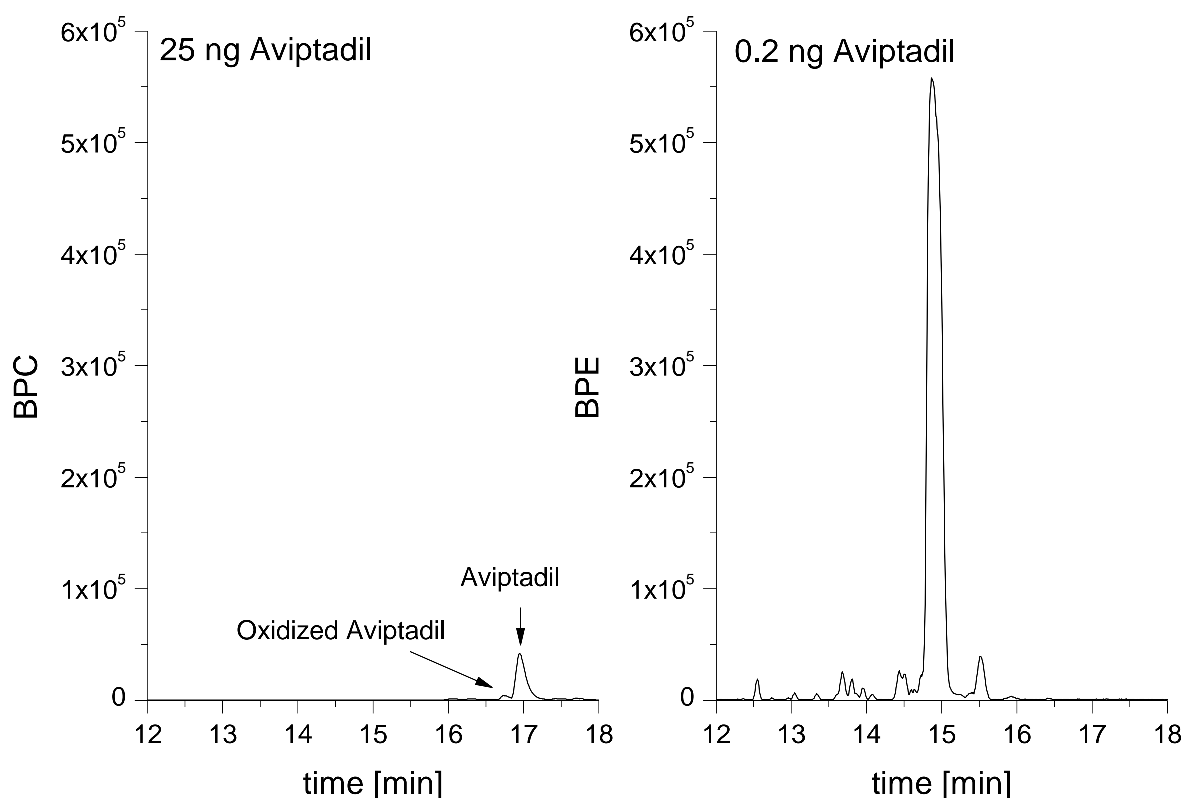
In Figure 3 a direct comparison between nanoRP-HPLC-MS and CE-MS is depicted, in both cases the same sample with the same aviptadil concentration of 25 µg/mL was injected. The total amount injected was in the case of nanoLC 25 ng (injection volume 1 µL) and in CE ~0.2 ng (injection volume ~7.7 nL). Although the injected aviptadil amount in CE was about 130fold less, the response area in the base-peak-electropherogram is ~4 fold larger and the signal height ~9 fold higher than in nanoRP-HPLC-MS. The sensitivity of our CE-MS method compared to our nanoLC-MS method could be estimated according to these values to be at least 500 fold higher.

Surprisingly, with the nanoLC method we detected a high amount of aviptadil oxidized at the methionine residue with a content of almost 7 % (see Figure 3). In CE the oxidized species was not

236 observed as individual peak but a small signal for it was found within the aviptadil main peak. The
237 difference between the detected amounts of oxidized methionine derivatives with both methods is
238 high. Possible explanations could be ion suppression effects by coelution with the main peak in CE
239 [14;16] or the observation may represent a methodological artifact in nanoRP-HPLC. The silica
240 surface could act as a catalyst for the oxidation or the matrix in LC might adsorb preferentially the
241 non-oxidized aviptadil API making the detected ratio between both species inconsistent with the real
242 values. Oxidations of cysteines during RP-HPLC analyses have already been described [17].
243 Importantly, the presence of oxidized methionine residues in proteins could result in a significant
244 decrease of hydrophobicity [18], increase the propensity for aggregation[19] and could impact drug
245 efficacy, safety, as well as antibody-drug half-life *in vivo*.[20] Since the monitoring of methionine
246 oxidation is currently routinely performed by peptide mapping RP-HPLC techniques, this point
247 seems important for drug quality analyses.
248 Figure 3 raises furthermore the question, why the signal intensity in our nanoLC-MS method is so
249 much smaller than in our sheathless CE-MS, reasons could be (i) the ESI-ionization efficiency (LC
250 flow 500 nL/min vs CE flow ~100 nL/min; different solvents and capillary tip geometries)[16; 21];
251 (ii) aviptadil substance losses in LC-MS because of unspecific adsorption and diffusion of the peptide
252 into the porous silica particles[22]. Further investigations should shed light on the real underlying
253 effects, whereas the latter point is of highly suspicion and often observed in RP-HPLC of biological
254 macromolecules.[22]
255
256 **Table 1.** Peak/mass-table of the base-peak-electropherogram in Figure 1 with interpretation, the mass errors of the
257 interpretation are indicated by a preceding 'Δ' sign.

Peak	Migration Time [min]	Peak Area %	Moniso. Mass [Da]	interpretation
A	10.3	0.06	1502.84	
B	11.6	0.55	1689.95	Ac-KQMAVKKYLNSILN-NH_2; Δ0.00
C	12.2	0.10	2207.27	
D	12.4	0.44	3099.65	DAVFTDNYTRLRKQMAVKKYLNSILN-NH_2; Δ0.00
E	12.5	1.07	3365.75	Aviptadil, acetylated; Δ0.01
F	12.6	0.27	1959.13	Ac-LRKQMAVKKYLNSILN-NH_2; Δ0.00
G	12.6	0.49	3186.68	SDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2; Δ0.01
H	12.7	0.21	1943.10 3336.74	
I	12.8	0.11	1949:96 3054.55	
J	12.9	0.14	3324.73	Aviptadil, deamidated; Δ0.00
K	13.0	0.73	3324.72	Aviptadil, deamidated -OH; Δ0.01
L	13.2	3.24	3323.74	Aviptadil conformer; Δ-0.01
M	13.2	<0.10	2984.62	HSDAVFTDNYTRLRKQMAVKKYLNS; Δ0.10
Main	13.5	90.99	3323.75	Aviptadil; Δ0.00
N	13.7	0.32	3209.69 3224.67	Aviptadil without 1 Asn; Δ0.02 Aviptadil without 1 Val; Δ0.01
O	14.0	0.85	3305.73	Aviptadil-H_2O, Δ0.01

258



259

260 **Figure 3.** Comparison between the nanoLC- and CE-MS method with regard to selectivity and sensitivity. In both cases,
261 the same aviptadil sample with a concentration of 25 µg/mL was applied. The total injected amount of aviptadil was
262 25 ng in case of nanoLC and ~0.2 ng in case of CE. On the y-axes MS detector signals for the base peaks are depicted in
263 the same scale for both samples (BPC=base peak chromatogram; BPE=base peak electropherogram). A calculation based
264 on the injected amounts and corresponding signal heights resulted in a ~500fold higher sensitivity of the sheathless CE-
265 MS method compared to the nanoLC-MS method. The impurity profiles observed with both methods are very different,
266 e.g. exhibiting a high amount of oxidized aviptadil in nanoLC without correlation in CE.

267

268 **3.4 Repeatability**

269 The repeatability was evaluated with two different capillaries by six subsequent injections of an
270 aviptadil solution with a concentration of 50 µg/mL each (total injected amount ~385 pg). Table 2
271 summarizes the results in detail, the relative standard deviation in percent (%RSD) for the migration
272 time is in both cases <1 %, a value acceptable for a CZE method. The %RSD values for peak height
273 and area are in both series <10 %, which is also acceptable for the ESI based detection and
274 comparable to the results gathered with the nanoRP-HPLC-MS method (data not shown). The
275 differences between the two capillaries/coatings were striking. This indicates problems concerning
276 the quantitative reproducibility of the results obtained with different coatings and/or capillaries.

277

278 **Table 2.** Base-peak-electropherogram evaluation of the repeatability data for the CE-MS method
279 (MT=Migration time; s=standard deviation).

	Capillary 1			Capillary 2		
Run	MT [min]	Height	Area	MT [min]	Height	Area
1	13.4	4.7E+05	2.7E+06	13.9	5.3E+05	5.1E+06
2	13.3	4.8E+05	2.7E+06	14.1	5.1E+05	4.7E+06

3	13.3	5.1E+05	2.9E+06	14.1	5.4E+05	4.9E+06
4	13.2	5.0E+05	2.7E+06	14.1	5.5E+05	4.6E+06
5	13.3	4.3E+05	2.3E+06	13.9	5.5E+05	5.4E+06
6	13.3	4.6E+05	2.5E+06	13.9	5.0E+05	4.3E+06
Mean	**13.3**	**4.8E+05**	**2.6E+06**	**14.0**	**5.3E+05**	**4.8E+05**
s	**0.1**	**0.3E+05**	**0.2E+05**	**0.1**	**0.2E+05**	**0.4E+06**
%RSD	***0.5***	***5.8***	***7.8***	***0.7***	***4.4***	***7.9***

280

281 **3.3 Limit of detection**

282 To determine the limit of detection (LOD), the aviptadil concentration was decreased while
283 keeping the injection volume constant at ~7.7 nL (1 % of total capillary volume), experiments were
284 done with capillary 1 (see Table 2). The base peak electropherogram of an aviptadil sample with a
285 concentration of 500 ng/mL (total injected amount 3.9 pg, ~1 fmol) aviptadil) resulted in a S/N ratio
286 for the aviptadil peak of 4. Additionally, the LOD was determined in an Extracted-Ion-
287 Electropherogram (EIE), which was reconstructed by addition of the different aviptadil charge states
288 (m/z values of 555.13 / 665.95 / 832.19 / 1109.25 with a window of ±0.5 Da). By this EIE
289 reconstruction procedure the LOD was determined to a concentration of 25 ng/mL, corresponding to
290 a total injected amount of 0.2 pg aviptadil (59 amol) with a signal-to-noise ratio of 3.
291 Comparing the determined LOD for aviptadil in the base peak electropherogram of ~1 fmol with
292 the LOD of 14 amol found by Faserl et al.[11] for angiotensin I, a ~nearly 100fold reduced sensitivity
293 was found. Both methods used the same instrumental setup with the same prototype sprayer and both
294 employed PEI coating. However, the examined analytes differ in size and pI. While for aviptadil four
295 different charge states were observed, only one charge state could be observed for angiotensin I.
296 Additionally, the isotopic signal distribution of aviptadil reduces the observed signal intensities.
297 Considering the different charge states during reconstruction of an appropriate EIE the determined
298 LOD of ~59 amol is in a similar range to that found by Faserl et al. for angiotensin.
299 With our nanoLC-MS system the LOD achievable in the base peak chromatogram was 10 ng
300 (3 pmol; 10 µg/mL aviptadil solution). This value is ~2500fold higher than the LOD found in CE-
301 MS. The comparison of the peak widths for aviptadil in the CE-MS electropherograms with the peak
302 widths in the nanoLC-MS chromatograms resulted in similar values (data not shown). This indicates
303 that the dilution effects are similar for both methods and confirms the results in Figure 3.
304

305 **3.5 Linearity & Range**

306 Several (> 4) prototypic capillaries/coatings were tested in this section without a consistent outcome.
307 Whereas the upper limit of quantitation (ULOQ) could be easily determined by the MS detector
308 saturation at concentrations >100 µg/mL, the exact determination of the lower limit of quantitation
309 (LLOQ) was not possible. Neither the usage of a S/N ratio >10, nor the definition of a defined
310 precision value, e.g. <5 %RSD, were successful because of fluctuating values. In all measured
311 linearity series we observed outliers. The best out of 4 independent data sets resulted in the equation
312 y=2.97E+5 x – 1.08E+5, obtained by linear regression, with a coefficient of correlation of R^2=0.965
313 at 7 different aviptadil concentrations (2.5 / 5.0 / 7.5 / 10.0 / 50.0 / 75.0 / 100.0 µg/mL), each level
314 tested in duplicates and evaluated by integration of the main peak in the base peak elctropherogram.
315 The evaluation via the peak heights of the same data set resulted in a coefficient of correlation of
316 R^2=0.989 for the CE-MS method. Overall we conclude, that the generation of quantitative data with
317 the current sheathless prototype is not easily to achieve. During the course of the linearity
318 measurements several problems became apparent: (i) the intensities were highly dependent on the
319 sprayer position and the distance between the ESI source and the CE tip, complicating quantitative
320 comparison between runs measured on different days; (ii) the PEI coating showed a high inter-

321 coating variability with divergent LOD's and LLOQ's, which was already described in the precision
322 chapter above; (iii) the handling of the fragile prototype capillary requires attention and accuracy as
323 the capillaries can break very easily not only at the porous tip, but also at the inlet side; (iv)
324 quantitative measurements could be also strongly dependent of the of the actual tip aperture in view
325 of a more or less flat tip end, this was not considered in this study [16].
326
327 **4. Conclusion**
328 The CE-MS method, based on the sheathless CE interface with the covalent basic PEI coating, is
329 able to analyze aviptadil qualitatively in respect to specificity. The determination of the peptide mass
330 by deconvolution subsequent to electrophoretic separation is straightforward and MS/MS
331 experiments can be used for structural analysis. Here the special mode of isCID-MS/MS was applied
332 and resulted in confirmation of 54 % of the aviptadil sequence. This approach can be seen as an
333 alternative under circumstances where conventional CID-MS/MS generates too few b- or y-ions for
334 sequence interpretation or where peptide mapping is to elaborate. The main motivation for this study
335 was to analyze orthogonality in separation principle of CZE vs. RP-HPLC for peptide analysis. Here
336 we expected to detect a different impurity profile, which was confirmed. None of the impurities
337 found with CE-MS could be found with LC-MS and vice versa. In this study more impurities were
338 detected by CE-MS than by our nanoLC-MS method (15 in CE vs. 2 in nanoLC). Even if
339 optimization of nanoLC eventually could result in more detectable peptides, we conclude, that the
340 analysis of RP-HPLC purified peptides is complemented by CE-MS by revealing a different impurity
341 profile. Most probably, the emergence of the completely new impurity profile in CE is a combination
342 of different selectivities, 'specific' peptide adsorptions in RP-HPLC and differences in ESI ionization
343 because of the different flow-rates in CE compared to LC. The detection of quite high amounts of
344 oxidized aviptadil in LC-MS could also reflect the hypothesis of selective peptide adsorptions, e.g.
345 the oxidized methionine is less hydrophobic than the unmodified API. Also the unexpected small
346 signal observed in Figure 3 for the main peak in LC could rely on peptide adsorptions effects besides
347 influences of higher flow-rates in LC. Further experiments are necessary to become a clarification
348 about the influences of the different effects. Nevertheless the relationship between the determined
349 Area% values for hydrophobic and less hydrophobic peptides in RP-HPLC analyses has to be
350 determined carefully and should be best assisted by other methods.
351 Whereas the qualitative analysis of specificity and the repeatability of the sheathless CE-MS were
352 actually satisfying and convincing, in the linearity section, problems with the prototype setting were
353 observed. The quantification strongly depends on the exact position of the CE tip in front of the ESI
354 orifice in both directions, the x- and y-axes. Furthermore, the quantitative comparison between runs
355 performed on different capillaries with different coatings (of the same nature) are problematic.
356 Obviously, CE-MS has potential to become a method as robust as LC-MS and by that paves the way
357 for a more complete characterization of therapeutic peptides.
358

359 ACKNOWLEDGMENTS
360 The authors would like to thank Beckman Coulter, especially Hans Dewald, Jean-Marc Busnel, Jim Thorne, Dona
361 Neloni and Dietmar Hansen for technical and theoretical support and supply of the prototypic capillaries. We
362 acknowledge Thomas Hoffmann and Holger Seelert for helpful discussions.
363 Work in our laboratory was funded by the BMBF and the European Community by EFRE C/4-PBT-400-4/2010.

364

365 Reference List

366 [1] Development trends for peptide therapeutics. 2010. San Diego, Peptide Therapeutics Foundation.

367 Ref Type: Report

368 [2] *Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins,* CRC Press, Boca Raton,
369 1984.

370 [3] M. Urbanek, L. Krivankova, P. Bocek, *Electrophoresis* **24,** (2003) 466-485.

371 [4] R. Ramautar, A. A. Heemskerk, P. J. Hensbergen, A. M. Deelder, J. M. Busnel, O. A. Mayboroda, *J.*
372 *Proteomics.* **75,** (2012) 3814-3828.

373 [5] M. Pioch, S. C. Bunz, C. Neususs, *Electrophoresis* **33,** (2012) 1517-1530.

374 [6] P. Pantuckova, P. Gebauer, P. Bocek, L. Krivankova, *Electrophoresis* **32,** (2011) 43-51.

375 [7] R. Haselberg, G. J. de Jong, G. W. Somsen, *Electrophoresis* **34,** (2013) 99-112.

376 [8] M. Moini, *Anal. Chem.* **79,** (2007) 4241-4246.

377 [9] J. M. Busnel, B. Schoenmaker, R. Ramautar, A. Carrasco-Pancorbo, C. Ratnayake, J. S. Feitelson, J. D.
378 Chapman, A. M. Deelder, O. A. Mayboroda, *Anal. Chem.* **82,** (2010) 9476-9483.

379 [10] R. Haselberg, C. K. Ratnayake, G. J. de Jong, G. W. Somsen, *J. Chromatogr. A* **1217,** (2010) 7605-7611.

380 [11] K. Faserl, B. Sarg, L. Kremser, H. Lindner, *Anal. Chem.* **83,** (2011) 7297-7305.

381 [12] A. A. Heemskerk, M. Wuhrer, J. M. Busnel, C. A. Koeleman, M. H. Selman, G. Vidarsson, R. Kapur, B.
382 Schoenmaker, R. J. Derks, A. M. Deelder, O. A. Mayboroda, *Electrophoresis* **34,** (2013) 383-387.

383 [13] H. H. Leuchte, C. Baezner, R. A. Baumgartner, D. Bevec, G. Bacher, C. Neurohr, J. Behr, *Eur. Respir. J.*
384 **32,** (2008) 1289-1294.

385 [14] D. A. Abaye, F. S. Pullen, B. V. Nielsen, *Rapid Commun. Mass Spectrom.* **25,** (2011) 1107-1116.

386 [15] F. Lottspeich, J. W. Engels, *Bioanalytik,* Elsevier, München, 2006.

387 [16] A. Schmidt, M. Karas, T. Dulcks, *J. Am. Soc. Mass Spectrom.* **14,** (2003) 492-500.

388 [17] X. Huang, J. Barnard, T. M. Spitznagel, R. Krishnamurthy, *Journal of Pharmaceutical Sciences* (2012)
389 n/a.

390 [18] V. N. Uversky, G. Yamin, P. O. Souillac, J. Goers, C. B. Glaser, A. L. Fink, *FEBS Lett.* **517,** (2002) 239-
391 244.

392 [19] M. C. Gaudiano, M. Colone, C. Bombelli, P. Chistolini, L. Valvo, M. Diociaiuti, *Biochim. Biophys. Acta*
393 **1750,** (2005) 134-145.

394 [20] C. Loew, C. Knoblich, J. Fichtl, N. Alt, K. Diepold, P. Bulau, P. Goldbach, M. Adler, H. C. Mahler, U.
395 Grauschopf, *J. Pharm. Sci.* **101,** (2012) 4248-4257.

396 [21] R. Kostiainen, T. J. Kauppila, *J. Chromatogr. A* **1216,** (2009) 685-699.

397 [22] *HPLC of biological macromolecules,* Marcel Dekker, New York, Basel, 2002.