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Cover image
Mercator gel (run by D. Ackermann at CUP) representing the award-winning CoFGE technology for standardized gel electrophoresis
Analyzing marker substances for Complex Regional Pain Syndrome (CRPS)

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Abstract

Less than 5% of patients develop Complex Regional Pain Syndrome (CRPS) after trauma, mostly after fractures. It is a painful syndrome characterized by a variety of clinical features including classical signs of inflammation and it can become chronic if not cured in the first few months. Likely, a number of pathophysiological mechanisms play a role in CRPS. The involvement of neuropeptides and anti-inflammatory lipid mediators has been suggested. Here, mass spectrometry (MS) was used to investigate these molecules in skin biopsies and serum with the aim of correlating their concentration with clinical parameters. High-end and in particular nanoscale MS identified peptides as well as fatty acids at the low fmol level. However, it also showed little tolerance for the chemical background so that a more robust capillary chromatography approach was preferentially used. Serum protease activity with a focus on angiotensin converting enzyme (ACE) was studied. Bradykinin (BK) was rapidly degraded to BK1-8 and BK1-5. The formation of lower BK1-5 levels was indicated in agreement with the hypothesis of reduced ACE-activity in CRPS.
Introduction

CRPS is a severe and often disabling syndrome, which develops after trauma in ~5% of all cases; most often after distal radius fractures [1]. CRPS is characterized by a variety of clinical features including spontaneous pain and hyperalgesia [2]. Increased neuropeptide release from peripheral nociceptors has been suggested as a possible pathophysiological mechanism triggering the symptoms [2]. Bradykinin (BK, Figure 1), for instance, participates in inflammatory processes, producing classical symptoms of inflammation such as redness, heat, swelling, and pain [3]. It was increased in blood samples from CRPS patients [4] and its decreased degradation by angiotensin converting enzyme (ACE) was thought to be involved in the pathophysiological mechanism [5, 6]. Other peptides of interest were calcitonin gene-related peptide (CGRP), substance P (SP), endothelin I (ET-1) and vasoactive intestinal peptide (VIP) (Figure 1) [7]. Moreover, resolvins (Figure 2), endogenous anti-inflammatory lipid mediators, are generated during the resolution phase of acute inflammation and emerging evidence points to a potent anti-hyperalgesic role in inflammatory pain [8, 9]. A detailed overview for these pro- and anti-inflammatory signal molecules including cytokines and peptidases and their analytical detection is given in [10].

Here, mass spectrometry (MS)-based investigations of both peptide and lipid marker substances in inflamed tissue (skin) and serum of patients vs. controls are described. The report is divided by task: 1) Extraction of the substances to be investigated from the respective biological matrix, 2) Analysis of resolvins, and 3) Detection of neuropeptides and monitoring of protease activity.

Bradykinin (BK)
RPPGFSPFR

Substance P (SP)
RPKPQQFFGLM-NH₂

Neurotensin (NT)
pQLYENKPRRPYIL

Vasoactive intestinal peptide (VIP) / VIP 1-12
HSDAVFDTNYTRLRKQMAVKKYLNSILN-NH₂

Endothelin 1 (ET-1) / ET-1 1-9
CSCSSLMDKECVYFCHLDIWW

Calcitonin gene-related peptide (CGRP) / CGRP 25-35
ACDTATCVTHRLAGLLSRSGVVKNFVPTNVGSKAF-NH₂

β-nerve growth factor (NGF) / β-NGF 1-9
SSSHPIFHRGEFSVCDSVSWVGDKTTATDIKGKEVMVLGEVNIINNSVFKQYFFETKCRD PNVPDSCRGIDSKHWNSYCTTTHTFVKALTMDGKQAARWRFIRIDTACVCLSRAVRRA

Figure 1: Increased neuropeptide release from peripheral nociceptors may be a possible pathophysiological mechanism triggering CRPS symptoms. Full sequences for peptides of interest are shown with target sequences marked.
Part 1
Extraction of marker substances from patient material

Biochemical indicators for an increased post-traumatic inflammation or an insufficient inhibition of the inflammation in CRPS should be preferentially studied at the location of the symptoms, namely in skin biopsies. Two skin biopsies are typically the maximum which can be expected from one patient at a time and for many tests such as zymography one is completely used up. Thus, we aimed for the double use of one biopsy for both the extraction of fatty acids and of peptides in order to save patient material. We first extracted the lipids with organic solvent and subsequently isolated the peptides (Figure 1.1).

Analyses were conducted following the general principle shown in Figure 1.2. First, the response of the instruments was determined with respect to sensitivity and accuracy using commercially available standards. In the second step, these standard substances were spiked to control material at defined concentrations in order to study their recovery and to find unexpected difficulties introduced by the biological material. Finally, the method was applied to patient samples.
Experiments

During the experiments, the declaration of Helsinki in its revised form of Oktober 1983, Venedig, was observed. The Ethics Commission of Landesärztekammer Mainz has allowed the clinical investigation and the collection of skin biopsies (No. 837.050.04 (4208), LÄK Rheinland-Pfalz). Inclusion criteria met the diagnostic criteria of the IASP (International Association for the Study of Pain; age > 18 years). Written informed consent was obtained from all subjects. Skin biopsies were collected intra-cutaneously after local anasthesia with 1 ml lidocain 2% at the most affected site and at the corresponding site of the contralateral extremity. Healthy control persons donated biopsies from the same sites. Breast skin tissue was used for method development. Subcutaneous fat was removed from the skin sample. Samples were frozen in liquid nitrogen and sent on dry ice from Mainz to Münster. They were stored at -80°C until further use.

Patient blood was drawn into EDTA-free S-Monovette® (Sarstedt). In case of the determination of endogenous BK, protease inhibitor-containing vials could be used in contrast to measurement of protase activity. In the latter case also the use of anticoagulants such as EDTA should be avoided as it is a Zn chelator and competes for Zn with ACE [11-12]. Following transport and handling at room temperature, samples were centrifuged and aliquoted for storage at -80°C. They were subsequently treated as illustrated in Figure 1.3. Several traditional methods for lipid extraction such as those of Folch [13] and Bligh and Dyer [14] have been tested (see also [15]). A major reason to ultimately perform resolvin extraction using the optimized protocol of Matyash et al. [16] and Giera et al. [17] was the fact that handling was much improved in the methyl-tert-butylether (MTBE) method as the organic layer was well accessible at the top (Figure 1.4).

Peptides were purified as shown in Figure 1.5. The larger peptides such as CGRP, VIP and ET-1 were tryptically digested to generate shorter target peptides for gas phase fragmentation analysis. This measure improved peptide recovery and identification confidence by matching experimental peptide fragment ions with MS/MS data obtained from the commercial standards. For the extraction of peptides, in particular BK, from serum or plasma, we followed recommendations by Aristoteli et al. [18]. The authors had compared several methods and found that both acetonitrile (ACN)-extraction (ACN:serum 2:1 v/v; 10:1 v/v) and ultracentrifugation showed best recoveries for low molecular weight peptides. In our case, the first method worked best when tested for BK (Figure 1.6). Thus, ACN was added to the serum at a ratio of 2:1 v/v and incubated for 30 min at room temperature at a shaker (1000 rpm). Subsequently, the sample was centrifuged for 10 min at 12000 x g. The supernatant was
lyophilised and redissolved in 10 µl 95:5 v/v H$_2$O:ACN, 0.1% formic acid (FA). Extracted peptides from skin biopsies were dissolved in the same way.

**Figure 1.3:** Treatment of skin biopsies for lipid extraction optimized after Matyash [16].
**Figure 1.4:** Comparison of lipid extraction methods. The advantage of the MTBE method is the convenient separation of the organic (org.) layer to the top. [16, 17]

MS experiments were performed using reversed-phase liquid chromatography (RP-LC) coupled to different types of mass spectrometers. Best sensitivity was achieved with nanoLC-MS/MS using nanoAcquity coupled to Q-TOF Premier or M-Class coupled to Synapt G2 Si (all from Waters Corp.). However, despite additional filtering steps, samples derived from these complex biological matrices tended to build up material in the nanoLC-system resulting in frequent clogging and instrument downtime. Therefore, a more robust capillary LC coupled to an iontrap instrument (Poroshell 120 EC-C18, Agilent HP1100, Bruker Esquire 3000) was used for most experiments. For measurement on the iontrap 5 µl were injected. AutoMS/MS on the doubly charged ion was used (scan range $m/z$ 100-1100). Commercial sources for chemicals and material are given in Table 1.1.

**Figure 1.5:** Peptides were isolated from the aqueous phase after lipid extraction.
Figure 1.6: MS/MS spectra for BK (50 pmol) recovery from 100 µl serum using ACN precipitation (see text and [18]). Dried extracts were redissolved in 10 µl 5% ACN containing 0.1% FA. LC-MS/MS was carried out with Esquire3000 on the doubly-charged BK ion (2 µl injection; note zoom area in spectra).

Table 1.1: Sources of neuropeptides, chemicals and material

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Ziptip® µC-18 Pipette Tips  ZTC18M690  Millipore
Oasis® HLB 1cc  186000383  Waters Corp.
S-Monovette®  02.1726.01  Sarstedt

Part 2
Resolvin MS-based analysis

As shown by other authors earlier, fatty acids can be detected with reversed phase chromatography coupled to MS, preferentially in negative ion mode [19-22]. Using positive ion mode, we demonstrated a detection limit in the low fmol range (Figure 2.1) even from methanol solutions; 0.1-0.3 ng fatty acid were sufficient for analysis. In negative ion mode, sensitivity was even better (low fmol / high amol range; shown for 15 pg in Figure 2.2). However, a limitation of the nanoLC technologies was their little tolerance towards chemical background of the sample so that the more robust capLC was alternatively used (Figure 2.3, 2.4).

Figure 2.1: Fragmentation analysis of RvD2 (500 fmol on-column) in positive ion mode using Q-TOF Premier and nanoAcquity (top trace MS scan). Water losses are indicated by arrows. The sodiated ion was more abundant than the protonated ion, but did not provide very specific MS/MS data (bottom trace). Therefore, for MS/MS the protonated ion which has lost one water molecule (m/z 359.2; middle trace) was used.
Figure 2.2: Fragmentation analysis of RvD1/2 (50 and 250 fmol on-column) in negative ion mode using Synapt G2 Si with iKey and MClass (Waters Corp.; 11 min runs, peptide BEH C18 130 Å 1.7 µm 150 µm x 50 mm iKey). a) Base peak chromatogram of MS and MS/MS (left) and MS peak for one scan (right).

b) Corresponding MS/MS data for 3 scans. Major fragment ions are labeled.
Figure 2.3: Esquire3000 MS and MS/MS spectra of RvD1. Top: MS spectrum of 100 pmol RvD1 spiked to skin biopsy; the extract was dissolved in 10 µl and 9 µl were injected for measurement. Bottom: two MS/MS spectra of precursor ion m/z 399.9, upper RvD1 recovered from biopsy, lower RvD1 standard (5 pmol on-column).

Figure 2.4: UV-chromatogram at 290 nm (HP1100) for RvD1 (19 min) and RvD2 (14.5 min) spiked to control skin and recovered at ~90 pmol.
Resolvin injection inhibits spontaneous pain, heat and mechanical hypersensitivity evoked by capsaicin and tumor necrosis factor (TNF)-α [23]. The detection of endogenous resolvins in body fluids or tissue is thus of interest but also a matter of debate. We could recover spiked resolvins from skin and detect it using LC/iontrap MS at pmol sensitivity (Figure 2.3; the instrumental detection limit of this set-up was 1 pmol resolvin standard on-column, recovery of 10 pmol resolvin spiked to water/ethanol 9:1 v/v was possible). For serum; spectra were obtained for 1/5 of the extract recovered from 100 pmol resolvin spiked to 100 µl serum. This volume was chosen as a compromise – extraction from more serum volume increased the danger of matrix accumulation on-column with low-flow chromatography.

So far, we have not been successful in finding endogenous resolvins neither in biopsies nor in serum even when using more sensitive analytical instrumentation. Neither did other authors working with state-of-the-art MS equipment [24-25] even when supplying fish oil containing nutritional supplements to probands. On the other hand, in an excellent quantitative study by Colas et al. [26] resolvins were measured in pooled serum of healthy volunteers at concentrations of 31 ± 7 pg/ml (RvD1) and 41 ± 14 pg/ml (RvD2) and in pooled human plasma at concentration of 2.6 ± 0.1 pg/ml (RvD1) [26]. The detected amounts however corresponded to the lower limit of the calibration curve or were even outside it. The multi-reaction monitoring approach was chosen to guarantee specificity but may have been hampered by co-isolated peaks. We have seen a lot of overlapping ions in lipid spectra as masses are often only 2 Da apart (unsaturation). MS/MS spectra were therefore populated by fragment ions derived from several precursors. We thus concluded that additional preparation and prefraccionation of serum lipids would be necessary to circumvent this effect. In any case, MS/MS fragmentation of fatty acids is characterized by water losses and few if any characteristic ions. As Figure 2.2b illustrates, RvD1 and RvD2 can hardly be distinguished apart from the ion at m/z 175 originating from the different location of the OH-groups. Moreover, variation in instrumental design and collision processes may cause a shift in fragment ion intensities which could be the reason for the differences in our MS/MS data and those shown by Colas et al. [26]. Other authors also claimed resolvin detection in blood but did not show experimental data [27, 28]. Murphy [29] elaborates in an interesting comment that there are inherent challenges in lipid analysis and that the results and conclusions which have been reached so far “are at odds with each other”. Working at the limit of detection, published data do not seem sufficiently robust so far.
Part 3
Neuropeptide MS-based analysis

Protease activity can be measured using established gelatinase activity assays and zymography [30, 31]. Those methods work quantitatively, but they require significant amounts of material. MS, in contrast, can detect peptide signals in the low fmol /high amol range and it offers the possibility for multiplexing. First results have been achieved investigating adrenocorticotropin und α-melanocyte-stimulating hormon in skin and skin cells in the context of protease activity (neprylisin, ACE [32, 33]. Although the low fmol MS detection limits for peptides are in general highly promising for biomarker monitoring, there are unexpected analytical challenges. SP and ET-1, for instance, showed fast adsorption to the vessel wall (glass, 5 min) which limited quantification as an LC injection cycle was as long as 3 min depending on the set-up. The negative potential of glass surfaces offers optimal conditions for electrostatic interactions with the positive charge of a peptide [34, 35]. It would be possible to affect the electrostatic interaction with the addition of salts [36, 37], but that is not an option in the RP-LC coupling. The recovery of ET-1 and VIP was not reliable even when tryptic digestion was used for the creation of target peptides. Disulfide formation may be partially responsible for this observation. The VIP target peptide was susceptible to in source fragmentation in the mass spectrometer preventing proper quantitation. Thus, we developed a MS-independent labeling method (either dabsylation or fluorescent labeling) to measure peptides resisting MS-based quantification with thin-layer chromatography (TLC, [38]). The LC-MS analytical work subsequently focused on BK and CGRP peptide 25-35. While CGRP could be measured intact (Figure 3.1), digestion improved detection from serum via MS/MS target analysis. In addition, β-nerve growth factor (β-NGF) was analyzed, because it was found to sensitize pain processing [39]. β-NGF tryptic peptide 1-9 was targeted. Figure 3.2-3.4 present data for peptide recovery from skin.

**Figure 3.1:** MS spectrum of CGRP (1 pmol) measured with Q-TOF Premier. Charge states are labeled.
Figure 3.2: MS spectra of β-NGF1-9. a/b) Recovered from biopsy (100 pmol β-NGF/biopsy), a) MS scan, [M+2H]^{2+} ion at m/z 534.4 b) MS/MS at m/z 534.4 c): MS/MS for trypsin-digested standard (10 pmol on-column).

Figure 3.3: MS spectra of CGRP25-35. a/b) Recovered from biopsy (100 pmol CGRP/biopsy), a) MS scan, [M+2H]^{2+} ion at m/z 588.8, b) MS/MS at m/z 588.8, c): MS/MS for trypsin-digested standard (10 pmol on-column).
Figure 3.4: MS spectra of BK1-9. a/b) Recovered from biopsy (10 pmol BK/biopsy), a) MS scan, [M+2H]^2+ ion at m/z 530.9, b) MS/MS at m/z 530.9, c) MS/MS for BK standard (2 pmol on-column).

Spiked peptides were recovered at the low pmol / high fmol level from both skin and plasma when measured with capLC-iontrap instrumentation (Figure 3.5, 3.6). However, in particular for CGRP and β-NGF recovery was not reliable. BK measurement appeared more promising and was studied in detail. Endogenous BK was detected with 10^3 to 10^4 fragment ion counts when purified from 1 ml plasma. It was, however, important when investigating BK to take precautions with respect to peptidase activity. In human blood BK is rapidly cleaved by ACE and carboxypeptidase N (CPN) [40, 41]. On the other hand, BK may still be formed from plasma kallikrein [42]. Modulation of BK signaling is largely determined by ACE which metabolizes the BK nonapeptide into BK1-7 heptapeptide and the BK1-5 pentapeptide [40]; degradation by aminopeptidase P (APP, cleaves position 1-2) and CPN (cleaves position 8-9) [41] (Figure 3.7). Interestingly, patients which took ACE-inhibitors when they were injured were more likely to develop posttraumatic CRPS [6]. ACE is known to degrade peptides (SP, ET-1, CGRP, BK [43]), and inflammatory cytokines [44]), but also activates inflammatory cytokines from inactive precursors [30] and thus participates in the regulation of posttraumatic inflammation.
Figure 3.5: Recovery of spiked peptides BK, CGRP_{25-35} and βNGF_{1-9} from 100 µl blood plasma. MS/MS spectra of a) BK1-9, 5 pmol spike, m/z 530.9, b) CGRP_{25-35}, 50 pmol spike, m/z 588.8, c) βNGF_{1-9}, 100 pmol spike, m/z 534.25

Figure 3.6: Recovery of BK spiked to 100 µl plasma. Detection at m/z 530.9 with iontrap.
In order to study BK degradation and protease activity in sera of CRPS patients, methods were developed to monitor levels of spiked BK and its products. BK1-8, BK1-7 and BK1-5 were investigated using capillary chromatography coupled to ion trap mass spectrometry (LC-MS; Agilent HP1100, Bruker Esquire3000) and nano and iKeyLC coupled to high resolution MS (Waters MClass & Synapt G2 Si). Experiments were conducted in 2 µl serum which degraded 50 pmol BK within 10-15 min at 37°C. Incubation was stopped by the addition of ice-cold acetonitrile and samples were filtered with Nanosep® Centrifugal Devices (cut off 3 kD, Pall Corporation; for filter tests see Figures 3.8 and 3.9) to protect low-flow chromatography set-ups. After lyophilisation samples were dissolved in 10 µl LC solvent A of which 5 µl were injected to capLC-iontrap and 1 µl to the nanoLC setup. Calibration curve and example spectra for iontrap-MS are shown in Figures 3.10 and 3.11, for Synapt MS in Figures 3.12 and 3.13.

**Figure 3.7:** BK cleavage sites

**Figure 3.8:** Recovery of 50 pmol BK1-9, BK1-8 and BK1-5 standards after cut-off 3 kDa filtration with Nanosep® Centrifugal Devices and Amicon®-Ultra Centrifugation Filters (Millipore) compared to measurement without filtration. Daughter ions m/z 904.3, 748.3 and 417.1.

**Figure 3.9:** Recovery of 50 pmol spiked BK1-9, BK1-8 and BK1-5 from serum after filtration with Nanosep®. Daughter ions m/z 904.3, 748.3 and 417.1.
Figure 3.10: Iontrap calibration curves for BK1-9, BK1-8 and BK1-5 on daughter ions $m/z$ 904.3, 748.3, and 417.1, respectively.

Figure 3.11: Iontrap MS/MS spectra for (from the top) BK1-9 ($m/z$ 530.9, 10 pmol on-column), BK1-8 ($m/z$ 904.3, 2 pmol on-column) and BK1-5 ($m/z$ 573.3, 2 pmol on-column).
Figure 3.12: Development of targets for TOF-MRM using Synapt G2 Si MS. MS/MS isolation was performed for the triply-charged ion of BK, the doubly-charged ions of BK1-8 and BK1-7 and the singly-charged ion of BK1-5.

Figure 3.13: BK1-8 calibration for iKey Synapt MS (50-500 fmol, 11 min gradient, m/z 452.74). Extracted ion chromatograms for m/z 642.3 and MS/MS spectra demonstrating excellent data quality across the concentration range.
Figure 3.14: BK1-9 degradation in control (n=3) and CRPS patient sera (n=12) measured with iontrap. MS/MS spectra (3 scans, intensities of fragment ions of BK1-9 m/z 904.3, BK1-8 m/z 748.3, BK1-5 m/z 417.1.
**Figure 3.15:** Exemplary time course for BK (50 pmol) degradation in 2 µl control serum shown for BK1-8 (MS/MS m/z 452.74).

**Figure 3.16:** Schematic representation of Synapt data (5 controls, 9 patients) for degradation of 50 pmol BK in 2 µl serum.
Results are shown in Figures 3.14 (iontrap), 3.15 and 3.16 (Synapt). While nanoLC-MS excelled in sensitivity and mass accuracy it suffered from a lack of tolerance to residual chemical matrix. We observed accumulation of material on the chromatographic column ultimately leading to clogging in-line frits and instrument shut-down. That was in part due to the unreliability of filtration units. Despite their use to create solutions devoid of molecules larger than 3 kDa, we on and off detected hemoglobin and albumin (67 kDa!) at large concentrations. This result explains the difficulties in the chromatographic separation, which was optimized for peptide separation, and it prevented us from the acquisition of larger sample sets. We found that even more unfortunate as the methodology worked equally well with freshly drawn capillary blood from a finger tip and thus allowed us to determine local peptidase activity at the site of inflammation.

Nevertheless, for the small sample set of 37 CRPS patients vs. seven healthy controls we learned, supported by the TLC-monitoring of BK products [38], that BK1-7 was transient while BK1-8 and BK1-5 provided reliable markers which were detectable after 1-2 min and leveled out at ~40-60 min [38, 45, 46]. The formation of lower BK1-5 levels in CRPS is in agreement with the hypothesis of reduced ACE-activity. However, the current patient cohort was both too small and too diverse to provide a clear picture.

**Conclusion**

LC-MS based analytical approaches shine for their sensitivity and specificity. Numerous methods have been developed worldwide for the detection of small and large components of biofluids and tissue. Methods which are established in clinical diagnostics use comparatively large amounts of biomaterial (e.g. 1 ml serum) and robust instrumentation although current research techniques can achieve detection limits in the pg or fmol range. While we have seen exceptional data for peptide detection from serum (high amol sensitivity) we also noted considerable interference by the chemical background. The reason for that is the reduced tolerance of nanoscale fluidic systems to solvent contamination and sample diversity. Therefore, we developed an alternative TLC-based approach for monitoring BK degradation by serum proteases [38]. The comparatively simple TLC method shone for its robustness and throughput and is currently further optimized for the measurement of capillary as well as venous blood.

**Acknowledgements**

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**References**


