Liquid Chromatography and Electrospray Mass Spectrometric Mapping of Peptides from Human Plasma Filtrate

Manfred Raida, Peter Schulz-Knappe, Gabriele Heine, and Wolf-Georg Forssmann
Lower Saxony Institute for Peptide Research, Hannover, Germany

We present a multidimensional approach to map the composition of complex peptide mixtures obtained as crude extract from biological liquids by (1) cation exchange chromatography and (2) subsequent microbore reversed-phase liquid chromatography and electrospray mass spectrometry coupling (LC-MS). Human hemofiltrate is an equivalent to blood and is used to obtain peptide material in large quantities from patients with chronic renal failure. The upper exclusion limit of the filtration membranes used results in a protein-free filtrate containing peptides in a range up to 20 ku. Using this unique peptide source, several thousand peptides were detected and an LC-MS data base of circulating human peptides was created. The search for known peptides by their molecular mass is a reliable method to guide peptide purification. (J Am Soc Mass Spectrom 1999, 10, 45–54) © 1999 American Society for Mass Spectrometry

The direct coupling of high-performance liquid chromatography (HPLC) and electrospray mass spectrometry has become one of the most important techniques for the analysis of protein and peptides mixtures in the last few years [1]. This technique is widely used in the analysis of mixtures containing limited numbers of peptides, e.g., enzymatic digests of proteins, to confirm primary structures or to search for posttranslational modifications [2]. It is furthermore used to identify the protein by peptide mass fingerprinting [3]. Other reports describe the analysis of complex biological samples which contain a large number of different peptides, such as the major histocompatibility complex (MHC) peptides [4].

These approaches are complementary to well-established two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) methods which are widely used to characterize complex protein mixtures, such as human heart proteins [5–7], or to create a protein disease data base of human body fluids [8, 9]. In contrast to 2D-PAGE, the combination of peptide separation by chromatography and mass spectrometry is ideally suited for the accurate and sensitive detection of small peptides.

This work focuses on the analysis of very complex peptide mixtures from biological liquids by liquid chromatography-mass spectrometry (LC-MS). It is the aim of the Lower Saxony Institute for Peptide Research (IPF) to discover novel peptide hormones from human blood [10, 11]. As a source for peptide purification, human blood ultrafiltrate, the hemofiltrate (HF), is acquired in large amounts. HF contains a large number of peptides in a wide concentration range and in a molecular weight range up to 20 ku. Following peptide extraction, several steps of chromatographic separation are performed. By specific biological assay systems, certain regulatory peptides have already been isolated from this source [12–14]. The use of HF overcomes many limitations of the direct analysis of blood plasma:

1. Larger plasma proteins like albumin, the globulins, and proteases are retained by the filter membrane, and they are therefore absent or significantly reduced in HF.
2. Hemofiltrate is easily available in large amounts. During each treatment about 20–30 L of HF are collected from each patient.
3. A wide variety of regulatory peptides are present in HF in concentrations equal to those in blood plasma [15].

These points make HF an ideal starting material for systematic mapping of peptides circulating in human blood.

It is generally accepted that the advance of the human genome project will lead to an increasing demand for the systematic characterization of processed gene products. This has led to the concept of "Proteomics" [16] as a new field of biomedical research. A proteome project attempts to identify proteins from a specific biological source, i.e., cell culture supernatant, tissues, etc. The blood is also a unique source for diagnostically and therapeutically relevant peptides

Address reprint requests to Dr. Manfred Raida, Lower Saxony Institute for Peptide Research Hannover, Feodor-Lynen Str. 31, D-30625 Hannover, Germany. E-mail: mraida@gmx.de

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[10]. Because of the overwhelming amount of plasma proteins, a systematic analysis of plasma peptides has not been performed to date.

The aim of the study presented here is the establishment of a map of the peptides of human blood as present in the hemofiltrate. Like the well known 2D-PAGE of the proteins from specific cell lines, from tissue or blood [17], this map expands the analysis of complex mixtures to lower molecular weights which are not covered by gel electrophoresis.

Experimental

Hemofiltrate was collected from patients with chronic renal failure and processed as described in detail [15, 18]. About 20 to 30 L of HF were collected from each patient during one treatment. In brief, using batches of from 800 to 10,000 L HF, the filtrate was cooled to 4°C, diluted to a final conductivity of 5.5 mS/cm, and adjusted to pH 2.7. After adsorption to a strong cation exchanger [Fractogel SP 650 (M), Merck, Darmstadt, Germany], peptides were eluted with 1 M NaCl and precipitated with ammonium sulfate (680 g/L). To remove residual plasma proteins, the pellet was resuspended in 2 L deionized water and subjected to an ultrafiltration step cellulose triacetate membrane, cut-off: 20 ku (Sartorius, Göttingen, Germany). Peptides in the ultrafiltrate from several batches (10,000 L HF) were then bound to a cation exchanger [Fractogel SP 650 (M) 6 cm i.d. × 20 cm] equilibrated with solvent A (0.1 M acetic acid, 20% methanol, pH 3.0) and eluted in a linear gradient from 100% solvent A to 40% buffer B (0.5 M acetic acid, 20% methanol, 1 M ammonium acetate, pH 5.5) in 50 min and from 40% buffer B to 100% buffer B in 10 min at a flow rate of 50 mL/min. Absorbance at 280 nm and conductivity were monitored and fractions collected every 2 min starting at minute 10. Fractions were lyophilized and aliquots taken for LC-MS analysis.

Pooled plasma from healthy volunteers (50 μL), hemofiltrate (50 mL), and the retentate and filtrate of the HF extract subjected to ultrafiltration (50 and 100 mL, respectively), were separated via a HiLoad 16/60 Superdex column (Pharmacia, Freiburg, Germany) after solid-phase extraction of samples using Sep-Pak C18 cartridges (Waters, Eschborn, Germany). The column was calibrated using a protein standard (human serum albumin 69 ku, ovalbumin 45 ku, horse myoglobin 17.8 ku, insulin 5.7 ku, and phenylalanine 147 u) and run at a flow rate of 1 mL/min using 50 mM NaH_2PO_4 (pH 6.8) containing 100 mM NaCl. Absorbance was monitored at 214 nm.

LC-MS analysis of the fractions collected after cation exchange chromatography was carried out on a microbore HPLC system consisting of an ABI-140B double syringe pump and an ABI-785 variable UV-detector (PE Applied Biosystems, Weiterstadt, Germany), with a capillary U-flow cell (LCPackings, Amsterdam, The Netherlands) equipped with a C-18 microbore column 1 × 100 mm, ODS-AQ 3 μm, 12 nm (YMC, Schermbeck, Germany) operated at a flow rate of 20 μL/min at ambient temperature. For all experiments, a solvent composition of 0.08% aqueous trifluoroacetic acid (TFA, v/v) (solvent A) and acetonitrile/water (9:1, v/v) with 0.065% TFA (solvent B, v/v) was used with a linear increase from 10% solvent B at a rate of 1%/min. The injection volume was 5 μL, containing an equivalent of between 100 mL and 1 L human hemofiltrate. The column eluent was directly transferred through a fused silica capillary with 50 μm i.d. and 45 cm length (Polymicro Technologies, Phoenix, AZ) to the electrospray interface of the mass spectrometer. The mass analysis was carried out on a Sciex API III (PE-Sciex, Langen, Germany), run in positive ion mode. The instrument was calibrated daily for the m/z range between 50 and 2400 by a mixture of polypropylene glycols (PPG), as recommended by the manufacturer. The data from the LC-MS analysis were collected over a range from m/z 400 to 2400 at 4 s/scan in 0.3 m/z steps with a spray voltage of 5000 V. The orifice voltage was concurrently scanned from 80 to 100 V with each mass scan. Nebulizer gas and curtain gas (both N_2) were set for optimal sensitivity during the calibration step. All other settings were optimized for approximately unit resolution throughout the range of interest.

Data were collected by the Tune program and manually analyzed by MacSpec 3.3 (both PE-Sciex, Langen, Germany). Automatic analysis of the raw data was also carried out with SHERPA 3.1.1 [19] using the primary search tools and the data file comparison option. The results from the automatic peptide mass determination were confirmed manually, as given in Results and Discussion. The search for known peptide masses was performed using the "Extract Ion" tool of MacSpec. At least three consecutive mass-to-charge ratio values of the individual peptide mass with a search range of ±1 u were used.

For unequivocal peptide identification, N-terminal sequence analysis of several peptides was performed after purification using the combination of ion-exchange and reversed-phase chromatography. Edman degradation was carried out with ABI 470, 473 or Procise 494 gas phase sequencers (PE-Applied Biosystems, Weiterstadt, Germany), using standard cycles and standard methods. About 6 to 15 amino acids were identified and a data base search was performed with FASTA [20] and available protein and nucleotide databases (SWISS-PROT; GenBank, dbEST, and others).

Results and Discussion

The analysis of HF has opened up a new way to address plasma peptides for a systematic approach using a peptide bank as starting material. Peptides can either be identified systematically, equivalent to the identification of proteins for 2D-PAGE as carried out in proteomics, or by isolation using a specific biological or immunological assay. In many cases, a very small amount of

peptide is obtained after multiple chromatographic steps. To obtain more material in a short time for further structural or functional analysis, the original purification procedure may be a limiting step. Using the molecular mass of the peptide of interest, isolated primarily, to analyze the LC-MS measurements from the whole starting material, it becomes possible to reduce the time of re-purification and increase the amount of peptide for further analysis. This peptide screening [21] is the chromatographic separation and precise mass determination of all peptides present in the complex mixture. The combination of parameters like elution time and precise molecular mass allows the follow-up of the isolation of a known peptide or of any peptide of interest.

The composition of plasma and hemofiltrate with respect to protein and peptide content and the preparation scheme for large-scale peptide extraction from HF is shown in Figure 1. Plasma proteins such as albumin and globulins account for the majority of the total protein mass (70 g/L, Figure 1A) which was determined by amino acid analysis of the plasma protein hydrolysates [15]. By arterio-venous hemofiltration [22], the larger proteins remain in the blood because of the 20 ku cutoff of the cellulose triacetate filter. The smaller molecular weight peptides are able to pass the filter and they are present in the HF (peptide mass 70 mg/L, Figure 1B). This leads to a reduction of a factor of 1000 for the larger proteins in the HF. As shown earlier, the concentration of peptide hormones in the filtrate equals the plasma concentration [15]. Because the amount of residual proteins, although greatly reduced in the HF by blood filtration, is still within a range of 50% of the total peptide/protein content in HF, we performed a second ultrafiltration to remove the remaining proteins. Figure 1C shows the size exclusion chromatogram of the retentate and Figure 1D of the ultrafiltrate. After extraction, precipitation and ultrafiltration, the peptides from 4800 L HF were separated into 35 fractions by preparative cation exchange chromatography (Figure 1E). These fractions serve in one of the IPF procedures as the basic approach to identify novel peptide hormone candidates. In the following, the analysis of these fractions by LC-MS is described.

Aliquots of all fractions from this 4800 L HF preparation were measured by LC-MS. Panel A of Figure 2 shows the reconstructed total ion chromatogram (TIC) of one fraction from cation exchange chromatography. The molecular masses indicate peptides which were determined manually using dominant mass-to-charge ratio series. Panel B shows the mass spectra of three selected areas from the TIC. In addition to the dominant mass-to-charge ratio values which represent the multiple charged ion species of indicated molecular masses, several other mass-to-charge ratio series can be detected in the mass spectra. This demonstrates the high degree of complexity of the sample. The combination of high resolution reversed-phase HPLC and mass spectrometry thus generates a great number of accurate and reliable data in every single LC-MS experiment.

The molecular masses in Figure 2 panel A were determined from mass spectra averaged over 10 to 12 single scans (equivalent 40–50 s). The masses depicted in B1, B2, and B3 were then selected for isolation and identification of the corresponding peptides from the cation exchange fraction. The three peptides as an example were identified as fibrinogen-Aα 414–442 (B1), thymosin-β4 3–33 (B2), and ubiquitin 1–72 (B3). The mass accuracy as calculated from the theoretical masses of isolated and identified peptides from these fractions is very high ($\leq$0.1%).

The manual analysis procedure was performed for all 35 LC-MS measurements and showed to be time consuming and operator dependent. For this reason, we also analyzed the LC-MS data with the "Ion Grouper" tool from SHERPA. This program allows the import of the raw data from LC-MS measurements and generates a table of molecular masses where multiple charged ion series are deconvoluted. An example of the SHERPA analysis of the LC-MS measurement shown in Figure 2 is given in Table 1. We allowed the program to find series with a maximum of $z = 12$, as taken from the mass-to-charge ratio series of the ubiquitin 1–72, and with a least two multiple charged states for a series. The mass-to-charge ratio values had to be in the same scan or in two neighboring scans. Gaps were not allowed. This setting allows the detection of peptides with molecular masses from 1000 to over 10,000 u. Peptides in the measurement that shows only single charged ions, most probably representing peptides below 1000 u, are not detected by this method. The threshold was set to a value determined manually from several parts of the TIC, to be in a signal-to-noise ratio greater than 3. From the LC-MS run shown in Figure 2, SHERPA detected 307 peptides in a molecular weight range from 800 to about 13,000 u. Manual confirmation of the molecular masses determined by automatic analysis revealed several limitations which led to artificial multiple ion series:

1. Taking only two to three highly charged states with $z = 8–12$, a molecular mass above 5000 u was calculated, marked with (1) in the Table. These molecular masses could not be confirmed by manual recalculation nor during rechromatography and peptide purification. As generally seen for peptides in this higher molecular mass range, more than four charged states per peptide are usually produced during electrospray ionization.
2. Mass-to-charge ratio series with more than three charged states were calculated in such a way that, in addition to the correct molecular mass, the deconvolution also gave separate masses corresponding to half or a third of the correct molecular mass. These artefacts could easily be detected since several mass-to-charge ratio values in one or neighboring scans were used for more than one peptide. These incidences are marked with (2).

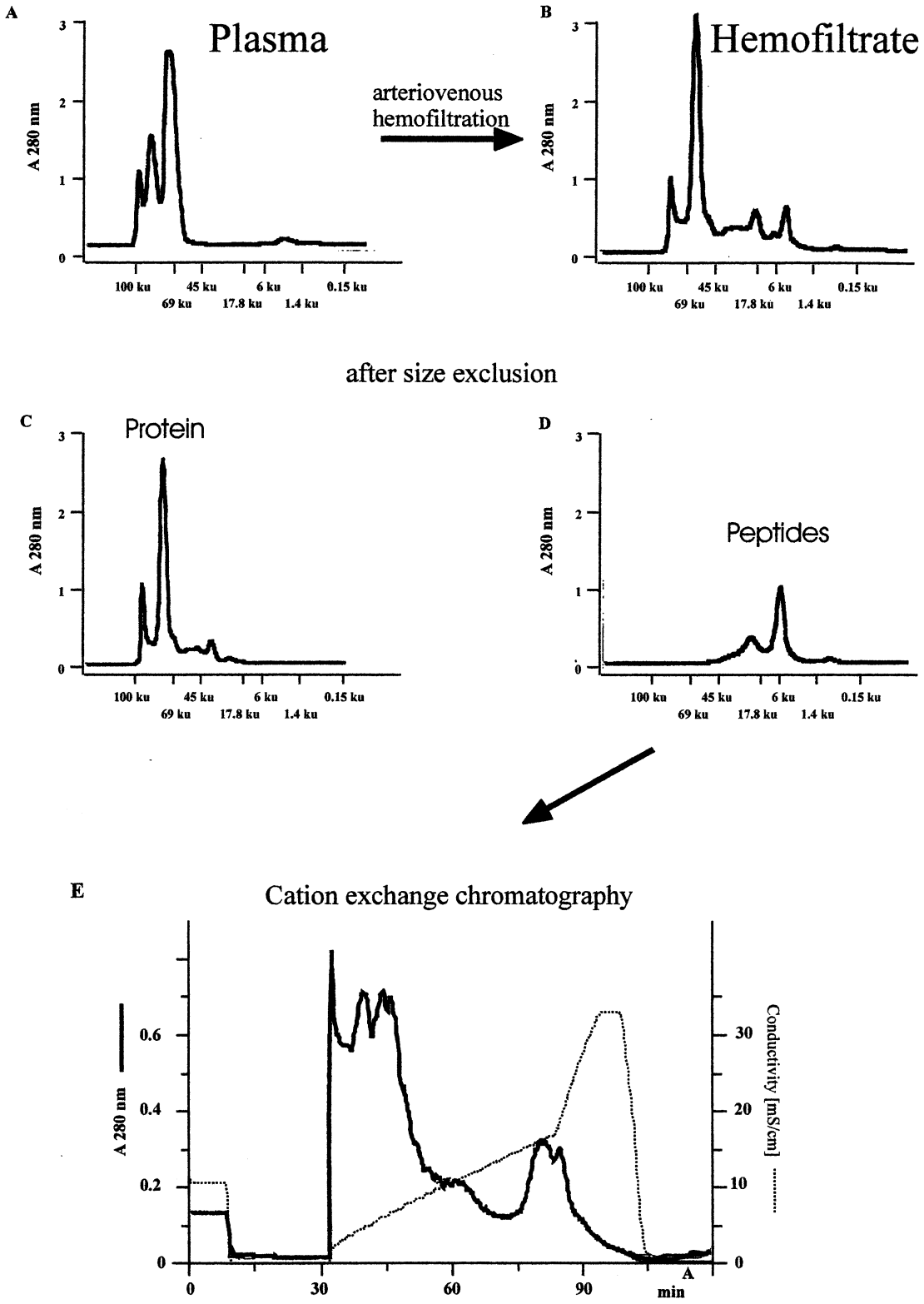

Figure 1. Subfractionation of HF. (**A**) Size exclusion chromatography (SEC) of 50 μL human plasma. The majority of the proteins are in the high molecular weight range. (**B**) SEC of 50 mL HF. After hemofiltration for removal of uremic substances, the content of plasma proteins in the filtrate is reduced by a factor >1000. (**C**) SEC of retentate of HF ultrafiltration (equivalent to 50 mL HF). (**D**) SEC of filtrate of HF ultrafiltration (equivalent to 100 mL HF). (**E**) Cation exchange chromatography of HF ultrafiltrate (equivalent to 4800 L HF).

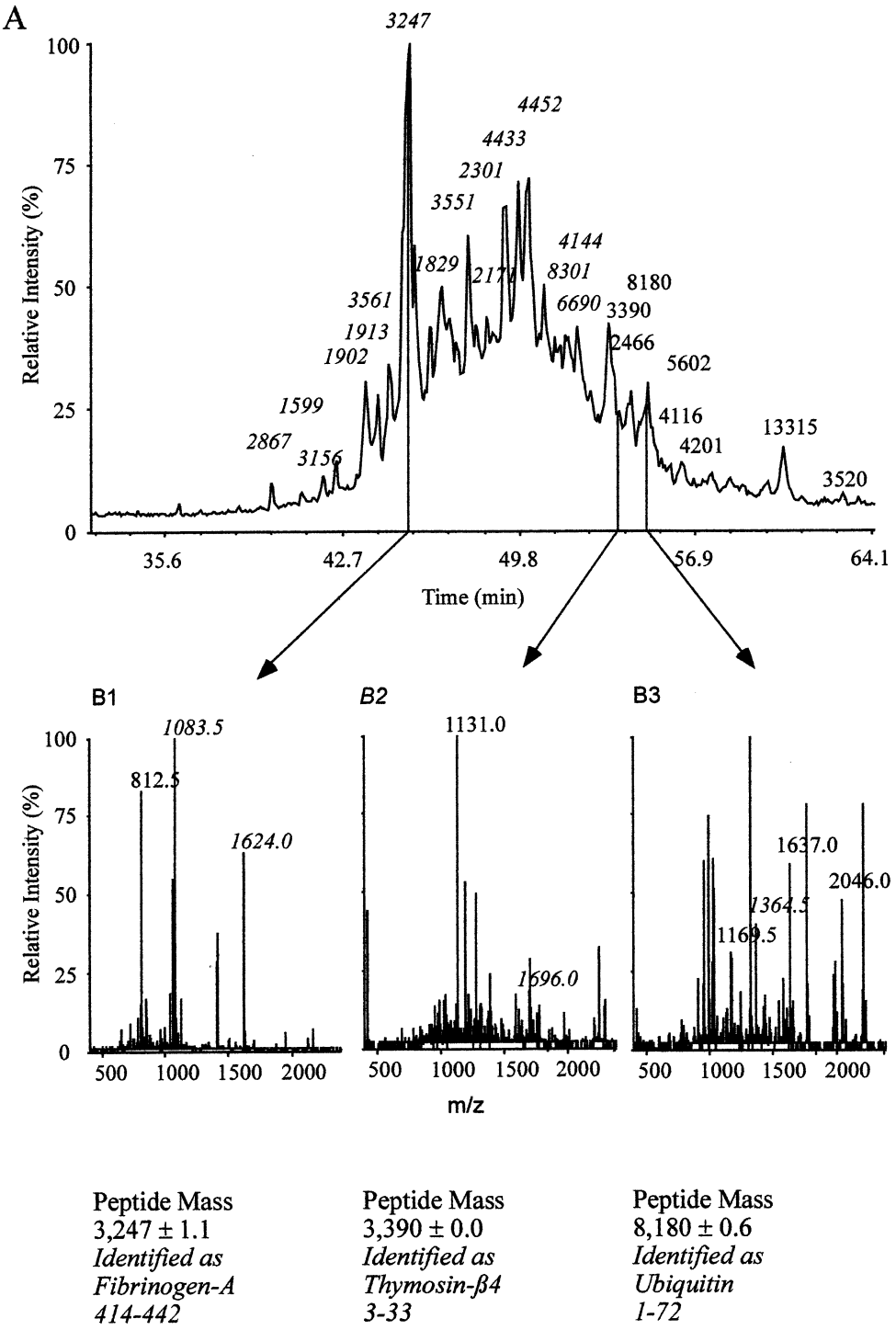

Figure 2. LC-MS analysis of fraction 17 from the cation exchange chromatography (Figure 1E). For details see the text. A: reconstructed TIC, B1–B3: mass spectra from three areas of the TIC, as indicated by the lines and arrows. The molecular masses of peptides and identification after chemical sequencing are given.

3. Charged states with low intensity were put together with high intensity ions for molecular mass calculations. Especially in complex samples with many charged states in one scan, this procedure leads to artefacts, marked as (3) in the table.

The automatic calculation succeeded in identifying all molecular masses determined by manual analysis (asterisk) and added several molecular masses with weak signal intensity or high scan complexity which were initially missed during the manual analysis (plus).

Table 1. Example of the molecular masses identified by automatic and manual analysis of the LC-MS run shown in Figure 2. An example of the original SHERPA result is given in A. B shows some selected molecular masses which have been detected by SHERPA. Calculated masses were selected to illustrate the artificial calculations as described in the text

A: Automatic and manual analysis of LC-MS runs

(*) Molecular mass identified manually and by the SHERPA

	MW	*m/z*	Intensity	Apex	Chrg.
	3246.91			638	
	3245.98	1624.00	262000	638	2
	3248.05	1083.69	315000	639	3
	3246.53	812.64	307000	638	4

(+) Molecular mass not identified manually, but by the SHERPA

	MW	*m/z*	Intensity	Apex	Chrg.
	5617.12			597	
	5619.61	937.61	14000	596	6
	5615.20	803.18	18000	597	7

(1) Highly charged ion series only

	MW	*m/z*	Intensity	Apex	Chrg.
	9956.45			637	
	9953.93	1107.00	23000	638	9
	9957.83	996.79	42000	637	10

(2) The same ions are calculated to result in different masses, like one half and one third of the original mass

	MW	*m/z*	Intensity	Apex	Chrg.
Incorrect	6665.16			848	
	6665.68	2222.90	23000	848	3
	6663.97	1667.00	10000	848	4
	MW	*m/z*	Intensity	Apex	Chrg.
Correct	13330.25			848	
	13331.35	2222.90	23000	848	6
	13333.03	1905.73	12000	848	7
	13327.94	1667.00	10000	848	8
	13327.72	1481.87	14000	849	9
	MW	*m/z*	Intensity	Apex	Chrg.
Incorrect	4443.33			848	
	4443.78	2222.90	23000	848	2
	4442.57	1481.87	14000	849	3

(3) Low and high intense ions are calculated together to result in a molecular mass which could not be confirmed manually

	MW	*m/z*	Intensity	Apex	Chrg.
	8493.87			634	
	8493.59	2124.41	33000	634	4
	8493.24	1699.66	22000	634	5
	8493.98	1416.67	226000	634	6

B:	Scan	Calculated mass	*z* values	Comment	Scan	Calculated mass	*z* values	Comment
	560	2867.0	2–3	*	634	4,247.1	2–5	*
	560	5733.1	5–6	1	636	11,954.3	11–12	1
	561	2293.3	2–3	+	638	3247.0	2–4	*
	561	3821.3	4–5	1	638	6493.2	3–9	2, 3
	588	2175.4	2–3	+	692	3399.1	3–4	+
	596	2464.5	2–3	*	692	4433.3	2–5	*
	597	4106.2	4–5	+	699	9940.7	9–10	1, 3
	597	5617.1	6–7	1	702	1191.1	1–2	+
	597	2406.9	2–3	+	705	4451.4	2–4	*
	597	6158.5	5–6	1	714	4038.9	3–4	+
	608	1901.8	2–3	*	714	13,468.6	10–11	1, 3
	612	3003.4	2–4	+	771	8180.0	4–9	*
	613	3132.3	2–4	*	776	9953.7	5–10	+
	613	7833.5	5–6	1	786	4115.8	3–4	*
	620	1912.6	2–3	*	792	5602.3	3–4	*
	626	6805.0	5–9	3	848	6665.2	3–4	2
	627	10,209.3	9–10	1	848	13,330.3	6–9	+
	628	2287.9	2–3	+	848	4443.3	2–3	2

The automatic analysis is of great help in mapping the peptide composition of complex mixtures, but results have to be interpreted in a critical manner. In the example shown here, 52% of the molecular masses detected by automatic analysis were confirmed manually. The remaining 48% turned out to be false positive molecular mass determinations which could be recognized immediately by careful analysis of the results. Different search settings, which are possible in the program, did not lead to significant changes in the reliability of the results.

To increase the accuracy of the programs used for automatic analysis of complex LC-MS measurements, the points given above should be recognized and incorporated into the search routines.

By identical analysis of all 35 LC-MS data sets, a comprehensive analysis of the peptides in HF was performed. More than 3000 distinct peptide masses have been identified from HF. The chromatographic profiles, TICs, individual scans, and the data tables from manual and automated deconvolution analysis from the basis of the method to search for individual peptide masses.

The use of LC-MS/MS for the identification of proteins and peptides is well recognized [23], but because of the fact that most peptides in HF are above the mass limit of MS/MS analysis, this technique cannot generally be applied here. Furthermore, the complexity of the HF fractions and the large number of coeluting peptides does not allow a specific peptide to be selected during the chromatographic separation for MS/MS analysis. Therefore, the peptides to be analyzed have to be purified to above 80% by consecutive chromatographic steps using conventional methods and characterized by Edman sequencing or off-line MS/MS. This characterization of the peptides of HF is currently being carried out.

The limited availability of native peptide hormones often represents a strong restriction for functional and structural analysis. It is therefore of great importance to find easy and fast ways to purify peptides from their biological source. The use of biological or immunological assay systems is often time consuming, expensive, and often not available for new peptides. The precise molecular mass in combination with other parameters, such as elution time, are strong tools to follow up the isolation of a known peptide from very complex mixtures.

During earlier experiments, a novel peptide of 36 amino acids (M_r 3928) was purified from HF and analyzed [18]. The sequence indicated that this peptide is related to β-defensins, which are supposed to exert antimicrobial activities. Therefore, this peptide was named hβ-defensin-1 (hBD-1). It was subsequently shown that hBD-1 plays an important role in epithelial defense mechanisms. Because of the size of the peptide and the presence of three disulfide bridges, the synthesis of this peptide proved to be difficult. To obtain reference material for synthesis and expression cloning as well as for the determination of the biological function, it became necessary to purify larger amounts of this peptide from HF. As shown in Figure 3, this can easily be done by retrospective analysis of the LC-MS data from HF preparations. All LC-MS measurements were analyzed for the presence of the ion series representative for hBD-1. The electrospray mass spectrum of the original peptide purification is shown in Figure 3A. Figure 3B depicts the TIC of the fraction in which the peptide's mass was found using the "Ion Extraction" tool. Only in this LC-MS measurement did all four selected mass-to-charge ratio values corresponding to the peptide's multiple ion series $z = 2$ to 5 show a signal intensity which was indicative for the presence of a peptide with the mass of hBD-1 (Figure 3C). The scans extracted from TIC are shown in Figure 3D. Using this approach, it was possible to detect and subsequently purify sufficient amounts for further analysis of structure and function.

The major advantage of this approach is that this method is extremely time saving. The entire search for any selected molecular mass is performed in 2 h of computerized analysis of all 35 LC-MS data sets. Further large-scale HF preparations are currently being mapped by LC-MS to allow the fast MS-guided detection of known peptides in our peptide fractions.

The approach presented here is used to select peptides according to their molecular mass for subsequent identification by partial sequence analysis. Table 2 summarizes the peptides identified from one fraction of the cation exchange chromatography. Four classes of peptides have been detected so far.

1. Known peptides, fragments of known peptides or proteins, and posttranslational variants and truncated peptides.
2. Novel peptides with homology to known peptides or protein families.
3. Novel peptides without homology to known peptide families, but with existing nucleotide sequences available in data bases (especially in dbEST).
4. Novel peptides without homology and data base entry.

Most peptides are fragments of larger plasma proteins, such as albumin, fibrinogen [24], and others [25], but some novel peptides, that were not present in any data base, have also been detected, such as hBD-1 or the novel chemokine HCC-1 [26]. Some of these sequences may already be available in the dbEST data base. With ongoing progress in human genome sequencing it can be expected that all peptide sequences will be easily linked to their mRNA and gene sequences within the next few years. The mass spectrometric analysis of complex peptide mixtures will become a strong tool to link "genomics" to "proteomics" as shown here, especially for circulating peptides from human blood.

Finally, it should be mentioned that LC-MS analysis of complex peptide mixtures does not allow the determina-

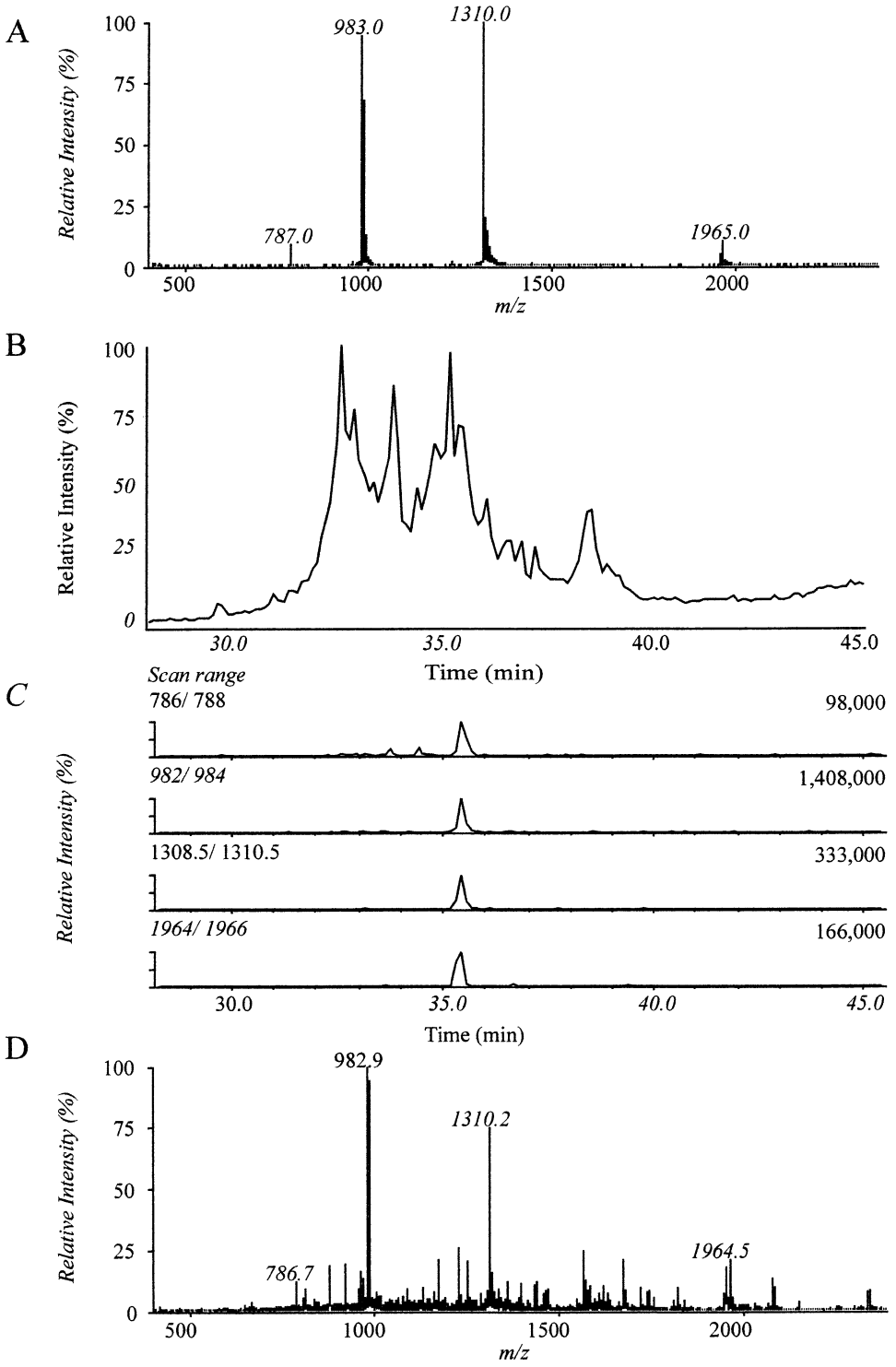

Figure 3. Search for a peptide with known molecular weight. (**A**) Mass spectrum of hBD-1, previously isolated from human hemofiltrate. The m/z values 787.0 $(M+5H)^{5+}$, 983.0 $(M+4H)^{4+}$, 1310.0 $(M+3H)^{3+}$, and 1965.0 $(M+2H)^{2+}$ represent the multiple charged ions of hBD-1 (M_r 3928). (**B**) Reconstructed TIC of the fraction in which the mass-to-charge ratio series of hBD-1 were identified. (**C**) Extracted ion scans for the four mass-to-charge ratio values representative for hBD-1. In scans 203–207 the intensities are increased in all selected mass-to-charge ratio regions. (**D**) Average mass spectrum from scans 200–210.

Table 2. Examples of regulatory peptides and fragments from serum proteins identified by mass spectrometry and chemical sequencing from different fractions of the cation exchange chromatography

Isolated and characterized regulatory peptides and active enzymes		
Name	Molecular mass	Comment
Angiotensin I	1295.7	
Uroguanylin 89-112	2,598.0	
hBD-1, β-defensin	3928.0	
Somatomedin-B	5003.0	
Vitronectin	5430.0–5,678.0	Different forms
HCC-1	8672	
Guanylin 22-115	10,337.0	
Pancreatic ribonuclease	14,600.0	Intact enzyme
Lysozyme	14,670.0	Intact enzyme
Uteroglobin	15,868.0	Heterodimeric protein

Purified and characterized fragments of serum proteins			
Protein	Number of fragments	Mass range	Comment
Serum albumin	28	349.2–8768.5	
Fibrinogen-Aα	34	1074.0–6955.4	
Fibrinogen-B	10	1263.7–4317.3	
β2-Microglobulin	19	570.5–11,729.0	Incl. full length protein
Cystatin C	15	932.5–6633.0	
α1-Antitrypsin	5	750.4–2595.4	
Thymosin-β4	5	2864.0–4247.4	
Ubiquitin	3	8181.0–8564.0	Incl. full length protein

tion of the concentration of specific peptides. Therefore, no data about peptide concentrations are given here.

Conclusion

The mapping of complex peptide mixtures from blood plasma was achieved by a combination of chromatographic methods and mass spectrometry. Several thousand peptide masses were determined in LC-MS measurements. The analysis of highly complex data sets has to be performed by a combination of manual and automated approaches. The computerized identification of peptide hormones in high numbers of peptide fractions is greatly facilitated by using the LC-MS data sets. Further software development is essential for the analysis and comparison of complex LC-MS data sets. Complementary to 2D-PAGE, the combination of chromatographic methods and mass spectrometry serves as a strong tool to develop proteome projects, especially for peptides.

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