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Detection and Quantification of Neurotensin in Human Brain Tissue by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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A method was developed for mass spectrometric detection of neurotensin (NT)-like immunoreactivity and quantification of NT in human brain tissue. The method is based on immunoprecipitation followed by analysis using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The identity of the major component of the immunoprecipitates as neurotensin was confirmed by fragment ion analysis on an electrospray ionization quadrupole time-of-flight instrument. MALDI-TOF-MS quantification of NT was achieved using stable-isotope-labeled NT as the internal standard, yielding an error of less than 5%. The method allowed detection of low-femtomole amounts of NT, starting from low-milligram amounts of lyophilized brain tissue. In addition to NT, several other peptides were detected in the purified samples, most of which, according to their molecular masses, corresponded to fragments of NT. The method is demonstrated with quantification of NT from human hypothalamus tissue, and a comparison is made with results obtained from competitive radioimmunoassay.

Neurotensin (NT) is a 13 amino acid peptide, present in several regions of the mammalian central nervous system, as well as in the small intestines and in the adrenal and pituitary glands of some species. In the brain, NT displays a heterogeneous regional distribution, with particularly high abundance in the hypothalamus. Extensive studies have shown that NT plays an important role in neuroendocrine regulation (see review in ref 1). Peptides with structural similarity to NT have been found in many species. One example is the mammalian neuromedin N,[2] which is synthesized as part of the same precursor protein as NT. Little is known about the biological roles of these NT-like molecules. From studies of rat brain tissue, there is evidence of region-specific processing of NT,[3] the significance of which largely remains to be elucidated. Radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and immunohistochemical techniques have provided the bulk of information on the regional distribution and abundance of NT-like immunoreactivity. Having provided clues to the biological role of NT, these techniques, however, lack the specificity to distinguish between modified forms of the peptide and between NT and structurally related peptides. In addition, they carry the risk of antibody cross-reactivity with other molecular species. Because of these general limitations of immunoassays, it is of interest to develop alternative techniques of higher specificity.

The advances in biological mass spectrometry (MS) over the past decade now permit sensitive detection, accurate mass determination, and structural analysis of a wide range of biomolecules (see reviews in refs 4−6). In combination with affinity-based purification methods, MS provides an alternative to commonly used immunoassays, with the ability to separately detect the components that constitute the immunoreactive species and gain structural information on these. The main advantage of the technique is the high specificity of detection; instead of environment-dependent properties, the intrinsic molecular masses of antigens, or MS/MS fragment ions thereof, are detected. Thereby, the problem of artifacts caused by antibody cross-reactivity and nonbiospecific retention of sample components, which are typically associated with RIA, can be overcome. The concept of using affinity-based purification techniques combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrom-

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etry (MALDI-TOF-MS) was introduced by Hutchens and Yip[7] and has since been developed and applied in several studies.[8−13]

For quantitative mass spectrometric analysis of biopolymers, electrospray ionization (ESI)-MS and secondary ion mass spectrometry (SIMS) have mainly been the techniques of choice (see reviews in refs 14 and 15). Because of several problems associated with quantitative MALDI-TOF-MS analysis (see below), the technique has so far mostly been restricted qualitative analysis. MALDI, however, has several advantages compared to other ionization techniques, including a very high sensitivity (low-femtomole levels of peptides can routinely be detected), ease of operation, and speed of analysis. Furthermore, this technique is very tolerant to sample contaminants, e.g., salts and detergents, which often impair analysis by ESI-MS. Therefore, the development of MALDI-TOF-MS as a quantitative tool has been the subject of an increasing number of reports.[14,16−28]

Problems associated with MALDI-TOF-MS quantitative analysis include low shot-to-shot reproducibility, sample heterogeneity, signal suppression, and nonlinear detector response. The use of internal standards and sample preparation techniques that yield more homogeneous crystalline samples[21,25,28,29] minimizes these problems. However, the use of standards that are chemically different from the analyte of interest may lead to unpredictable discrimination effects in the sample/matrix cocrystallization and desorption/ionization processes.[17] Furthermore, these effects may be influenced by the solvent system used and by other molecules present in the sample. The use of stable isotopes as internal standards for mass spectrometric quantification, on the other hand, has the advantage of the standard being chemically equivalent to the target molecule, at least if ^{13}C or ^{15}N labels are used, because they do not lead to a significant isotope effect. Thus, the risk of discrimination between analyte and standard in the purification and sample preparation steps are eliminated. Stable-isotope-labeled compounds have been used extensively as internal standards for peptide quantification in biological extracts by SIMS (see reviews in refs 15 and 30), and their use was reported by Duncan et al. in the MALDI-TOF-MS quantification of 3,4-dihydroxyphenylalanine and acetylcholine.[19]

In the present study, a method was developed for immunoaffinity purification of NT-like immunoreactive species in brain tissue extracts and subsequent detection of the immunoreactive species by MALDI-TOF-MS. The use of a crude polyclonal antiserum for the immunoprecipitation allowed detection of several truncated forms of NT, in addition to the full-length peptide, in human hypothalamus extracts. Quantification of the known biologically active form of the peptide NT 1−13 was achieved by spiking the crude tissue extracts with synthetic stable-isotope-labeled NT in which six carbon atoms in Leu^3 were replaced by ^{13}C ($[^{13}C_6]$NT), giving a molecular mass increment of 6 Da. The use of a standard that is chemically equivalent to the target molecule eliminates errors due to different affinities of the antigen and standard for the antibody and different ionization efficiencies in the MALDI process.

MATERIALS AND METHODS

Materials. Neurotensin, α-cyano-4-hydroxycinammic acid (HCCA), and ammonium bicarbonate were purchased from Sigma (St. Louis, MO), trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL), HPLC grade acetonitrile (ACN) was obtained from Merck (Darmstadt, Germany), acetic acid was purchased from Prolabo (Paris), and Protein G Sepharose 4 Fast Flow was purchased from Pharmacia LKB Biotechnology AB. Presiliconized microcentrifuge tubes (0.65 mL) from Corning Inc. (Corning, NY) were used for sample handling. $[^{13}C_6]$Neurotensin was synthesized by the SynPep Corp. (Dublin, CA). Millipore water was used for all solutions. Postmortem brain tissue was snap-frozen in liquid nitrogen, homogenized, and stored at −80 °C prior to the preparation of extracts. The use of this material was approved by the Ethics Committee for Medical Research of the University of Göteborg. NT polyclonal antiserum was a gift from Dr. E. Theodorsson, Department of Clinical Chemistry, Linköping University Hospital, Linköping, Sweden.

Methods. *Preparation of Protein Extracts from Brain Tissue.* Lyophilized hypothalamus samples (100−200 mg) were suspended in 1 M acetic acid (4 mL), and the suspensions were boiled for 15 min. After being cooled to room temperature, the samples were centrifuged for 20 min at 20000*g* at room temperature. The supernates were lyophilized and redissolved in 0.5 mL portions of buffer containing 10 mM PO_4^{3-}, 140 mM NaCl, and 3 mM KCl, pH 8.0 (PBS). Insoluble materials were removed by centrifugation

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at 20000*g* for 10 min. Aliquots of 20 μL were stored at −20 °C for further analyses.

Determination of Total Protein Concentration in Extracts. The total protein concentration in the brain tissue extracts was determined with a BCA Protein Assay Kit (Pierce). The kit is based on the use of bicinchoninic acid (BCA) for the colorimetric quantification of total protein.

Quantification of Neurotensin by Competitive Radioimmunoassay (RIA). Lyophilized tissue extracts were reconstituted in 0.05 M sodium phosphate with 6.7 mM EDTA, pH 7.4, containing 0.25% human serum albumin (HSA) (Behringwerke AG) and 500 kIU/mL aprotinin (Bayer, Leverkusen, Germany). The same buffer was used to dilute antiserum, tracer, and calibration concentrations of neurotensin. Antiserum samples (Hc-8; final dilution 1/100 000) were first incubated with calibrator or extract for 24 h at 4 °C and then with the HPLC-purified tracer for another 24 h. Bound and free [^{125}I]NT species were separated using Pharmacia Decanting Suspension (PDS; Pharmacia Biotech).

Preparation of Antiserum for Immunoprecipitation of Neurotensin from Tissue Extracts. NT antiserum was prepared from NT-immunized rabbits according to the procedure of Theodorsson-Norheim and Rosell.[31] The antiserum was purified by Protein G affinity chromatography on a Protein G HiTrap column (Pharmacia Biotech) following the recommendations from the manufacturer. NT-immunoreactive fractions were pooled and dialyzed against 50 mM sodium phosphate buffer, pH 7.4, lyophilized, and subsequently reconstituted in PBS.

Immunoprecipitation of Neurotensin. A 20 μL portion of brain extract was incubated with 100 μL of NT antiserum diluted 1/100 (v/v, relative to the original serum volume) in PBS at 4 °C overnight. A 15 μL washed Sepharose G beads suspension (Pharmacia) was added, and the sample was rotated slowly for 2 h at room temperature. The beads were spun down by brief centrifugation in a table centrifuge. All liquid was removed using a flat pipet tip (GT-250-2, Rainin) connected to water suction and quickly replaced by 400 μL of PBS. This washing procedure was done in triplicate. After the third wash, 20 μL of 0.5% TFA (v/v) was added to the beads. The sample was vortexed for 10 s and spun down in a table centrifuge. The supernate was transferred to a fresh vial using a long and narrow pipet tip (GELoader, Eppendorf), flattened at the outlet, to prevent beads from entering the tip. The elution step was repeated once using 20 μL of 0.1% TFA (v/v). The pooled supernates (approximately 40 μL) were stored at −20 °C for further analyses.

Mass Spectrometric Analysis of NT Immunoreactivity. The immunoaffinity purification procedure yielded samples that were diluted and contained phosphate salts. It was thus necessary to concentrate and desalt the samples prior to MALDI-TOF-MS analysis.[32] These steps were performed with a simple reversed-phase purification procedure described previously.[33] After loading of a sample onto the column and a subsequent washing step, the analyte molecules were eluted with 50−100 nL of matrix solution directly onto the MALDI target. A 10 μL quantity of sample (of a total of 40 μL) was used for sample preparation, using α-cyano-4-hydroxycinnamic acid (HCCA) as the MALDI matrix. The samples were analyzed on a Voyager Elite Biospectrometry Workstation (Perseptive Biosystems) in the reflector mode, using delayed ion extraction. Internal calibration was performed using the NT 1−13 signal, the identity of which was confirmed by nanoESI-MS (see below).

NanoESI-MS/MS Analysis. Nanoelectrospray MS/MS analysis was performed on a quadrupole time-of-flight mass spectrometer (QSTAR, PE-Sciex, Toronto, Canada). An immunoaffinity-purified sample, corresponding to 10 μL of crude hypothalamus extract, was loaded onto a reversed-phase microcolumn,[33] desalted, and eluted directly into the nanoelectrospray needle (Protana, Odense, Denmark) using 2 μL of the spray solution (60% methanol, 5% formic acid).

Quantification of Neurotensin by MALDI-TOF-MS. The following sample preparation, spectra acquisition, and data-processing procedures were used for all quantitative analyses, including the construction of the standard curve. The crude tissue extracts were spiked with synthetic [$^{13}C_6$]NT as the internal standard (for amounts, see below), after which immunoprecipitation was performed as described above. The MALDI sample preparation was modified to yield homogeneous sample spots. Analytes were eluted (with an HCCA matrix solution) from the reversed-phase column (see above) onto a preformed microcrystalline layer of HCCA, prepared by deposition of a 0.5 μL saturated solution of HCCA in acetone/0.1% TFA, 100/1 (v/v). Positive ions were analyzed on a Bruker Reflex II mass spectrometer (Bruker Daltonik, Bremen) in the reflector mode, using delayed ion extraction (ca. 350 ns delay time). Ions below m/z 1400 were deflected. The desorption laser was slightly defocused, and the detector voltage was set to 1.5 kV (0.1 kV lower than the current normal value for the detector). Spectra acquisition and processing were carried out automatically using AutoXecute software (Bruker Daltonik). The general principles of this automatic acquisition routine are described in the Discussion. The ability to sum up spectra in a controlled manner and the suitability of the AutoXecute routine for automatic data acquisition were the reasons that the quantitative measurements were performed on this instrument. A routine within XTOF software (Bruker Daltonik) was used for automatic data processing: The acquired spectra were smoothened slightly (three points), after which the first three isotope signals of NT and [$^{13}C_6$]NT were labeled and their intensities written to a report file. Further data processing was carried out in Excel (Microsoft): The ratio of the NT to [$^{13}C_6$]NT signal intensities was measured as the average intensity of the first three monoisotopic peaks of NT divided by the average intensity of the first three monoisotopic peaks of [$^{13}C_6$]NT. With this ratio, the NT concentration in the sample was calculated from a standard curve. The construction of the standard curve is described in the following.

Commercial, pure NT was dissolved in PBS at a concentration in the range of 1 nmol/μL, after which the concentration was determined by UV absorption at 280 nm. This stock solution was diluted to 1 pmol/μL, and aliquots thereof were stored at −20 °C. Synthetic [$^{13}C_6$]NT was dissolved in PBS and its concentration adjusted so that the signal intensities of [$^{13}C_6$]NT and NT were roughly equal when an aliquot of the [$^{13}C_6$]NT solution mixed

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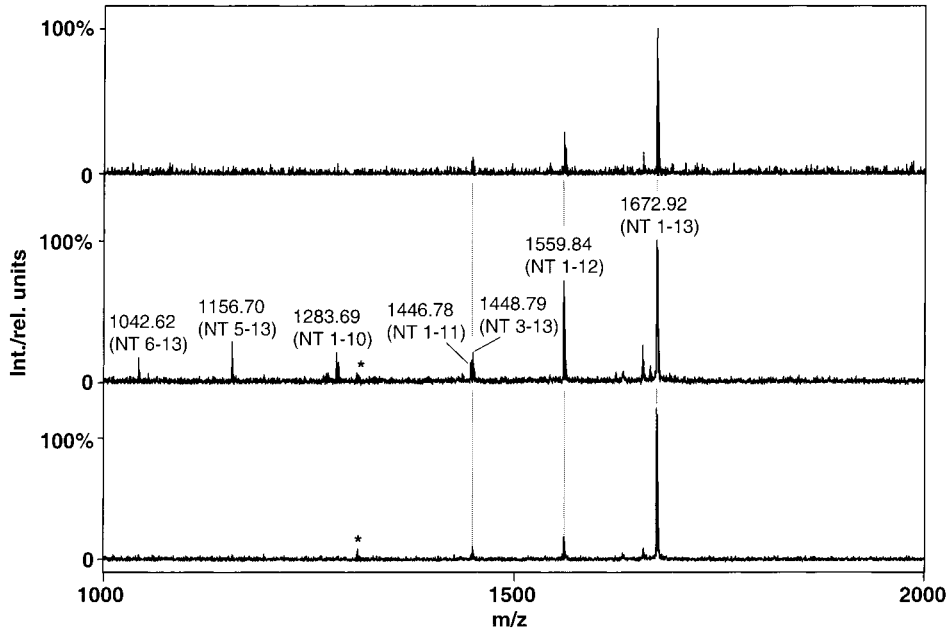

Figure 1. MALDI-TOF mass spectra of NT immunoprecipitates from hypothalamus samples from three individuals, indicating a varying abundance of partially degraded NT. Signals are assigned that match NT cleavage products within a mass accuracy of 50 ppm and labeled according to the position of the peptide in the NT amino acid sequence. The signals marked with asterisks are background signals also present in blank experiments (in the absence of antiserum).

with an equal volume of NT stock solution was analyzed by MALDI-TOF-MS. A series of PBS solutions were prepared, all containing the [$^{13}C_6$]NT solution diluted 1/200 and an NT concentration varying from 0.1 to 20 fmol/μL. An aliquot of each solution was analyzed by MALDI-TOF-MS as described above, and a standard curve was constructed by plotting the ratio of the NT to [$^{13}C_6$]NT signal intensities as a function of the NT concentration (Figure 4). Note that it is not necessary to know the exact concentration of the [$^{13}C_6$]NT standard; only, the concentration of the NT standard solution used for constructing the calibration curve is needed. For quantification of NT in tissue extracts, an aliquot of the extract was spiked with an equal volume of the [$^{13}C_6$]NT solution diluted 1/200. After MALDI-TOF-MS analysis, the NT concentration was obtained from the standard curve.

RESULTS

Immunoprecipitation of Neurotensin. Figure 1 shows the MALDI-TOF mass spectrum obtained from hypothalamus samples of three individuals after immunoprecipitation. In addition to the NT 1−13 signal (m/z 1672.92 monoisotopic peak), several other signals were observed, the masses of which correspond to various truncated forms of the peptide, as indicated in the figure.

NanoESI MS/MS Analysis of NT Immunoreactivity. The identification of NT as the major component in the immunoprecipitates, observed by MALDI-TOF-MS as a singly charged molecular ion at m/z 1672.92, was confirmed by nanoESI tandem mass spectrometry. A triply charged ion at m/z 558.32 was observed in the MS^1 spectrum. This species was selected for a collision-induced dissociation (CID) (Figure 2), yielding doubly charged fragment ions at m/z 578.86, 643.38, 724.91, and 836.97, corresponding to the doubly charged y''_9, y''_{10}, y''_{11} and y''_{13} ions of NT (Roepstorff−Fohlmann−Biemann notation).

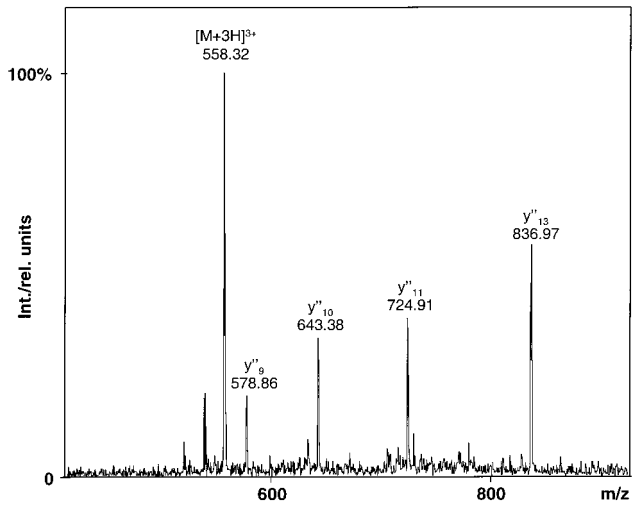

Figure 2. NanoESI daughter ion spectrum of $[M + 3H]^{3+}$ (m/z 558.32) from human hypothalamus tissue after immunoprecipitation, yielding a series of C-terminal doubly charged y″ ions confirming the identity of the isolated peptide as NT 1−13.

Quantification of Neurotensin by MALDI-TOF-MS. The quantification procedure relies on the ratio of the analyte (NT) to the standard ([$^{13}C_6$]NT) signal intensites. Relative signal intensities between analytes in a sample, however, vary greatly for single-shot spectra. Figure 3a shows four 10-shot spectra obtained from the same sample of a mixture of NT and [$^{13}C_6$]NT. As indicated in the figure, the signal intensity ratio (NT/[$^{13}C_6$]NT) varies more than 20%. Averaging of a large number of single-shot spectra from a sample is thus necessary to establish a stable ratio between the intensities of analyte and standard. Figure 3c shows the cumulative signal intensity ratios as a function of the number of accumulated spectra, obtained from the same sample. Sums of 10 single-shot spectra were acquired in succession. The intensity ratio was

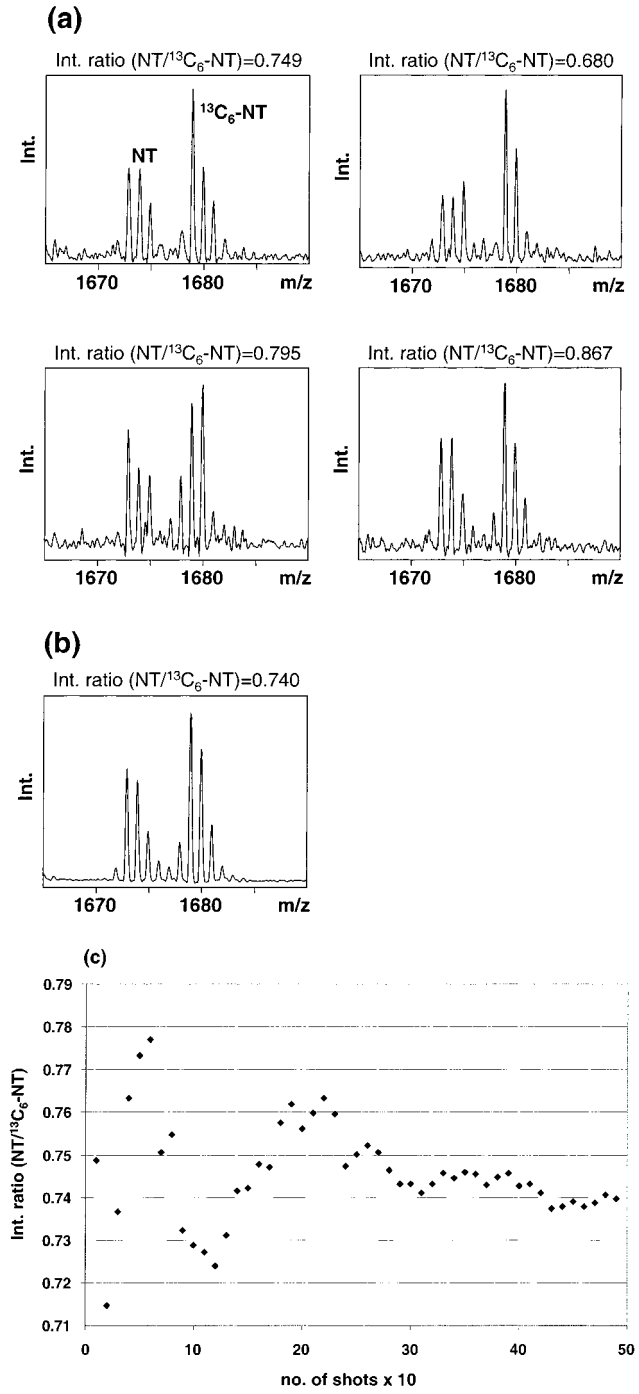

Figure 3. (a) Four 10-shot MALDI-TOF mass spectra acquired from a sample containing 20 fmol of NT and 25 fmol of [$^{13}C_6$]NT. The measured signal intensity varies more than 20%. (b) Spectrum of the same sample after accumulation of 400 single-shot spectra. (c) Signal intensity ratios as a function of the number of accumulated 10-shot spectra. After acquisition of a total of 400 single-shot spectra, a variation of less than ±2% was obtained.

calculated and plotted for each addition of a 10-shot spectrum. As indicated by the diagram, a precision of ca. 2% was obtained after accumulation of 400 single-shot spectra, resulting in the sum spectrum shown in Figure 3b. This number of accumulated spectra was used for the subsequent quantification experiments.

Standard Curve for NT Quantification. The standard curve for NT quantification is shown in Figure 4. The correlation

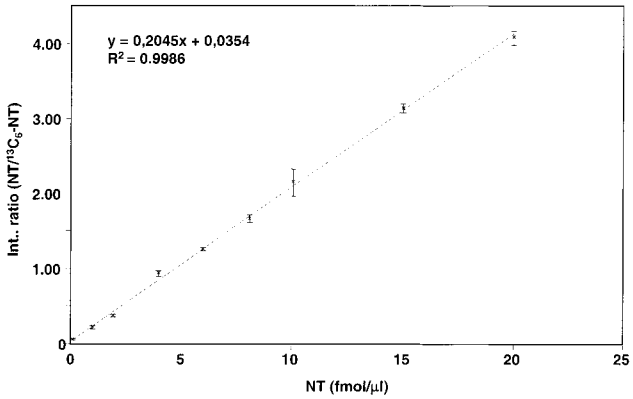

Figure 4. Standard curve for NT quantification. The relative standard deviation of the slope was 1.42%, and the standard deviation of the intercept was 0.0283. The vertical lines indicate the 95% confidence interval (±1.96 standard deviations around the mean) for each concentration. Three measurements were performed for each concentration.

Table 1. NT Concentrations Measured in Human Hypothalamus Extracts from Nine Individuals by MALDI-MS and RIA

		NT concn			
		MALDI-MS		RIA	
sample no.	tot. protein concn, µg/µL	fmol/µL	fmol/µg of protein	fmol/µL	fmol/µg of protein
1	1.6	4.6	2.9	5.2	3.3
2	2.5	3.6	1.4	6.2	2.5
3	2.4	4.0	1.7	6.4	2.7
4	2.8	6.5	2.3	8.3	3.0
5	2.6	9.0	3.4	11.0	4.2
6	2.2	4.9	2.2	8.1	3.7
7	2.5	3.5	1.4	6.1	2.5
8	1.9	3.3	1.7	4.0	2.1
9	2.2	3.7	1.7	5.5	2.5

coefficient (R^2) was calculated to be 0.9986 with a relative standard deviation of the slope of 1.42%. The standard deviation of the intercept was 0.0283. The vertical lines indicate the 95% confidence interval for each concentration. Three measurements were performed for each concentration.

Recovery of Neurotensin. A 100 fmol sample of NT in 20 µL of PBS was subjected to immunoprecipitation. The sample was subsequently spiked with an equal volume of the [$^{13}C_6$]NT standard diluted 1/200 in PBS. A 5 µL quantity of the sample was analyzed by MALDI-MS. Another sample containing equimolar amounts of NT and [$^{13}C_6$]NT was also analyzed, and the recovery of NT was calculated as the signal intensity ratio (NT/[$^{13}C_6$]NT) of the first sample divided by the signal intensity ratio of the latter sample. The recoveries were 45% and 68% from two immunoprecipitation experiments.

Quantification of NT from Human Hypothalamus Tissue. The NT concentration was determined in hypothalamus extracts from nine individuals by MALDI-MS and by RIA. The results are summarized in Table 1, and a graphical comparison of the results obtained by the two techniques for the measured NT concentration relative to the total protein concentration in the extracts is shown in Figure 5. The measured NT levels are similar those previously reported for extracts from human hypothalamus.[34]

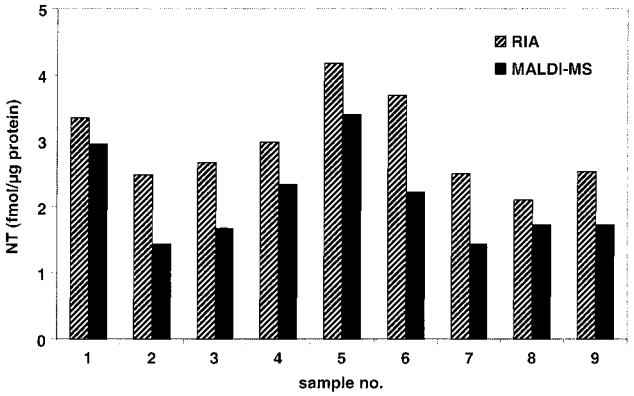

Figure 5. Comparison of NT concentrations in hypothalamus extracts from nine individuals, as measured by MALDI-MS and RIA, respectively.

DISCUSSION

Detection of NT-like Immunoreactivity. In the present study, we have investigated the use of crude NT antiserum after Protein G purification for affinity isolation of NT and subsequent mass spectrometric detection. This simplified approach is advantageous because affinity purification of antibodies directed toward small peptides is hampered by a reduced antibody affinity for a small antigen bound to a solid support. Furthermore, affinity purification of the antiserum carries the risk of yielding a subset of antibodies, whose high affinity for the immobilized antigen is not reflected for the antigen free in solution. Antibody cross-reactivity, which was expected to present problems, can easily be monitored since the antigen is detected at its characteristic mass.

A benefit of the method that is probably to some extent the result of using a crude polyclonal antiserum containing several antibodies directed toward different epitopes of the NT sequence is the possibility of detecting modified forms of the antigen. The detection of signals with m/z values corresponding to C-terminally truncated NT fragments (Figure 1) is consistent with previous studies of rat brain tissue, where two endopeptidases have been shown to cleave specific peptide bonds in the biologically active C-terminal of the peptide.[35] The abundance of these signals was found to vary greatly among the samples analyzed. The concentrations of the corresponding peptides, however, cannot be assessed from these experiments; i.e., their isolation is dependent on their unknown affinity for the antiserum and on the presence of other molecules that may compete for antibody binding. A comprehensive characterization of the NT-like reactivity will require further studies and is beyond the scope of this paper.

It is, however, inevitable that the presence of these partially degraded forms of the antigen will influence the immunoreactivity as measured by RIA to an extent depending on their abundance and affinity for the antibody. Consequently, a high level of NT-like immunoreactivity measured by RIA may not necessarily imply a high concentration of NT but may reflect a high abundance of partially degraded NT and/or other molecules that cross-react with the antiserum. Mass spectrometry, on the other hand, detects molecules at their characteristic m/z values or, by MS/MS fragment ions, whereby the technique offers the unique possibility of accurately quantifying a given target molecule.

Quantification by MALDI-TOF-MS. In the present study, a synthetic stable isotope of the target molecule was added to the crude tissue extracts and used as the internal standard for quantification. The standard, being chemically equivalent to the target molecule, will bind with the same strength to the antibodies as the target molecule and undergo the same quantitative losses in the subsequent purification step. Furthermore, it will be incorporated in the same way in the matrix crystals and be ionized with the same efficiency. These properties of the standard eliminate the risk of errors occurring through variation in the sample handling, preparation, and ionization.

Using a stable isotope as internal standard, however, does not automatically yield reproducible and precise quantification of the analyte by MALDI-MS, mainly due to two factors: the ion statistics and the detector response. It is not known how many ions of a given type typically hit the detector in a MALDI-MS experiment. The observation that the relative signal intensities of an analyte's isotopic envelope in a single-shot spectrum may deviate significantly from the predicted isotopic distribution suggests that the number may be rather small. When the laser is slightly defocused, analyte molecules are desorbed from a larger sample area.[18] This adjustment leads to a slight decrease in resolution but increases the total ion yield, thus improving the ion statistics.

The dual microchannel-plate detector used for ion detection is gives rapid and sensitive results but displays a large variation in signal response upon the impact of a given number of ions. Furthermore, the regeneration time for individual channels following the impact of ions is in the range of milliseconds, and thus the detector gives a significantly weaker response during this time period, following the impact of a large ion package. Keeping the laser fluency at a minimum and using a lower detector voltage than normal minimizes the effect of such detector saturation. The latter adjustment somewhat compromises the detection sensitivity but results in less electronic noise and a more reproducible detector response. Furthermore, more reproducible results are obtained after the ion-source and detector power supplies are switched on for 30 min.

Accumulation of the large series of spectra necessary for precise quantification is tedious. Therefore, the data acquisition was automated using the AutoXecute software. The software controls the MALDI probe movement and adjusts the laser intensity using real-time fuzzy logic feedback control.[36] After the acquisition of a small number of single-shot spectra, the control algorithm evaluates signal intensity and mass resolution of the base peak. It then regulates the laser fluence to keep the ion signal intensity within the dynamic range of the detection system, while maintaining high mass resolution. When the signal intensity is decreasing on one spot on the sample, it moves the MALDI probe a small distance to irradiate a fresh spot. In this manner, a large number of spectra with high resolution and similar signal intensities are summed from several positions on the sample. In the present study, the analysis time for each sample was 4−5 min;

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however, faster data acquisition systems are now available, which would reduce this time significantly. Following acquisition, data processing was carried out automatically with XTOF software, using a macro to calibrate the spectra, assign the individual isotope signals from NT and [$^{13}C_6$]NT, and report the signal intensities. The automatic acquisition worked well for most samples; however, for a few samples of very low signal intensity, manual data acquisition was necessary.

The choice of a suitable active matrix compound and a suitable sample preparation procedure are important parameters in the MALDI-MS analysis. For quantification purposes, 2,5-dihydroxybenzoic acid (DHB) was expected to be the best matrix because it allows long series of single-shot spectra of similar signal intensity to be obtained from a single position on the sample. With the immunoaffinity-purified brain samples, proper crystallization with DHB was, however, often impaired, probably due to sample contaminants, e.g., lipids. HCCA was found to be a good choice and is significantly more tolerant toward contaminants than DHB. For the automated quantitative analyses, a highly homogeneous sample preparation was required, which was achieved by using a combination of the reversed-phase nanocolumn preparation procedure[33] and the sandwich method.[32] With the use of a saturated solution of HCCA to elute the analytes from the column, the 45% ACN did not dissolve the preformed microcrystalline layer of HCCA crystals. This sample preparation technique, which is a modification of the fast-evaporation method,[37] has previously been reported to be of advantage for quantitative purposes because it yields a good spot-to-spot and intersample signal reproducibility.[29]

Mass Spectrometric Quantification versus RIA. As can be seen in Figure 5, there is a correlation between the results obtained by the two techniques. The consistently lower values obtained by MALDI-MS are in accordance with prediction because the latter technique only quantifies the molecular species NT 1−13, whereas the RIA values include contributions from partially degraded NT and possibly also other structurally related peptides. It is noteworthy that the intrasample differences between the NT concentrations measured by the two techniques are not uniform for the samples. The differences indicate a varying abundance of NT fragments and/or other NT-like molecules relative to NT in the samples.

The RIA technique is unsurpassed with regard to detection sensitivity ca. 100 amol for the NT assay compared to low-femtomole amounts detected by the MALDI-MS quantification procedure. An important difference between the techniques is that, for RIA, the sample is dissolved in a buffer containing a high amount of protein (0.25% HSA), whereas the MALDI-MS analysis requires purified antigen and the absence of protein background. This requirement is a general problem for the detection of low amounts of peptide and protein dissolved in pure solvent because of large losses due to adsorption to surfaces, e.g., those of sample vials and pipet tips. Improved sample handling and the use of low-adsorbing materials are likely to lower the detection limit.

Another difference between the two techniques regards the antibody consumption, which for RIA is low (100−1000 times lower than that for MALDI-MS) because the antigen concentration is measured by the competition for the antibody between the antigen and a radioactive tracer. The MALDI-MS quantification procedure described here, on the other hand, detects the antigen directly and, therefore, requires a molar excess of antibody for efficient affinity capture. Thus, the practical usefulness of the method depends on the ability to use inexpensive cheap and concentrated antiserum such as the crude rabbit antiserum used in this study.

CONCLUSIONS AND PERSPECTIVES

Mass spectrometric analysis in combination with affinity capture of NT from brain tissue yields more specific information than traditional immunoassays such as RIA. It allows modified forms of the antigen to be distinguished and, thereby, permits molecule-specific quantification without the risk of artifacts associated with the other techniques. The described method could be extended to also include stable-isotope-labeled NT fragments, which would allow not only precise quantification of NT fragments in a sample but also determination of the relative affinity of the antiserum for the different fragments, thereby providing a method for quantitative epitope mapping.

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