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Analysis of neuropeptide Y and its metabolites by high-performance liquid chromatography–electrospray ionization mass spectrometry and integrated sample clean-up with a novel restricted-access sulphonic acid cation exchanger

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Abstract

A novel restricted access cation exchanger with sulphonic acid groups at the internal surface was proven to be highly suitable in the sample clean up of peptides on-line coupled to HPLC–electrospray ionization (ESI)-MS. Neuropeptide Y (NPY) and several of its fragments in plasma were subjected to the sample clean-up procedure. The peptides were eluted by a step gradient from the restricted access column, applying 10 m*M* phosphate buffer pH 3.5 from 5 to 20% (v/v) of acetonitrile with 1 *M* NaCl and transferred to a Micra ODS II column (33×4.6 mm). The separation of the peptides and their fragments was performed by a linear gradient from 20 to 60% (v/v) acetonitrile in water with 0.1% formic acid and 0.01% trifluoroacetic acid in 4 min at a flow-rate of 0.75 ml/min. An integrated and completely automated system composed of sample clean up–HPLC–ESI-MS was used to analyze real life samples. The sample volumes ranged between 20 and 100 μl. Peaks due to the fragments NPY 1–36, 3–36 and 13–36 in porcine plasma were identified by ESI-MS. The limit of detection was in the 5 nmol/ml range. The total analysis required 21 min and allowed the direct injection of plasma.

Keywords: Restricted-access media; Sample handling; Column switching; Neuropeptides; Peptides

1. Introduction

Biologically active peptides are attracting remarkable interest in different areas such as pharmacology, chemistry and biology. Neuropeptide Y is a peptide neurotransmitter occurring in both the central nervous system and peripheral tissues. It acts as a vasopressor agent and affects ingestive behavior, depression and anxiety. The sequence of neuropeptide Y is given Fig. 1. Table 1 shows some physico–chemical characteristics of porcine neuropeptide Y and its fragments. The endogenous neuropeptides are present in biological tissues and fluids and, typically, immunoassays are used for the analysis of neuropeptide Y in complex matrices. However, immunoassays are frequently developed for a single analyte, where multiple analyte determination is called for. In

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H-Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH_2

Fig. 1. Sequence of porcine neuropeptide Y.

addition, cross-reactivity with unknown peptide fragments, similar peptides as well as with matrix components compromises quantitative aspects. Therefore, alternative analytical methods need to be developed. In the last two decades, work has been undertaken on the isolation, purification and detection of different types of neuropeptides [1]. Isolation and identification of neuropeptides in body fluids and tissue extracts have been performed by combining size-exclusion chromatography (SEC) and mass spectrometry (MS) [2] and by capillary electrophoresis (CE) coupled with MS [3]. Reversed-phase HPLC and affinity chromatography with immunoassay and radioimmunoassay (RIA) facilitated the simultaneous measurement of numerous neuropeptides in body fluids such as plasma and cerebrospinal fluid [4–6]. All the techniques require an efficient sample clean up prior to separation to enrich the target peptide and to eliminate proteins and other contaminants.

Sample preparation in the determination of neuropeptides in biological fluid ranges from simple techniques, such as extraction and centrifugation, to solid-phase extraction (SPE) and ultrafiltration [7].

Table 1
Characteristics of neuropeptide Y and its fragments[a]

Peptide	Molecular mass	Isoelectric point, p*I*
Neuropeptide Y (porcine)	4254.7	7.3
Neuropeptide Y (3–360	3994.4	7.3
Neuropeptide Y (13–36)	2983.8	9.6
Neuropeptide Y (18–36)	2457.8	10.4
Neuropeptide Y (22–36)	1904.2	10.9

[a] Theoretical values calculated from MassLynx version 3.2 (Micromass, UK).

Among these, SPE has become the most applied sample clean-up technique [8]. New selective SPE packings, such as mixed-mode and restricted access materials (RAMs) and immunosorbents and molecularly imprinted polymers have been studied in this context [9,10]. Although HPLC–MS and HPLC–MS-MS have been applied for the determination of a wide variety of pharmaceutical compounds using SPE off-line as a clean-up and concentrating step [11–13], SPE can be coupled directly to HPLC [14,15]. The HPLC-integrated sample preparation with RAMs is based on the complete non-adsorptive size exclusion of macromolecules, e.g., proteins, and on the simultaneous adsorption of the target molecules at the inner surface of the RAMs [16–18]. The on-line mode of sample clean-up using a RAM as a precolumn and column switching offers a number of advantages: (i) it allows repeated direct injection of untreated biofluids, (ii) provides on-column enrichment of analytes, (iii) enables matrix-independent high analyte recovery and (iv) improves precision, accuracy and sensitivity as well as giving a high throughput and low costs per analysis [19,20]. Integrated sample clean up with a RAM has been mainly applied for the analysis of low-molecular weight compounds, e.g., drugs and their metabolites in biological fluids [21–29]. However, few studies into the use of RAMs in the clean up of peptides and protein samples have been reported [30].

The key focus of the present work is to apply a novel restricted-access sulphonic acid cation exchanger and to perform an integrated sample clean up with neuropeptide Y and its fragments. Firstly, the conditions for the sample clean-up and reversed-phase (RP)-HPLC separation of neuropeptide Y were assessed in off-line mode. Secondly, optimization of the conditions in an integrated sample clean up–RP-

HPLC–MS system was applied for the analysis of neuropeptide Y and its metabolites in plasma.

2. Experimental

2.1. Reagents and samples

Potassium dihydrogenphosphate monohydrate, orthophosphoric acid (85%), sodium chloride, acetonitrile GR (99.9%), formic acid and trifluoroacetic acid were supplied by Merck, Darmstadt, Germany. All buffer salts were of analytical reagent grade. Water was taken from Milli-Q system (Millipore, Bedford, MA, USA). Neuropeptide Y (porcine) (>97%), neuropeptide 3–36 (porcine) (>98%), neuropeptide 13–36 (porcine) (>96%), neuropeptide 18–36 (porcine) (>99%) and neuropeptide 22–36 (porcine) (>97%) were purchased from Bachem, Bubendorf, Switzerland. All peptides were obtained as the trifluoroacetate salts. Porcine plasma samples were from AstraZeneca, Mölndal, Sweden. All solutions were degassed with helium (15 min) immediately prior to use.

2.2. Sample clean-up

For the sample clean-up and the reversed-phase separation in the off-line mode we used an HPLC equipment consisting of two pumps, a high pressure mixing chamber, a variable absorbancy wave length UV detector (supplied by Bischoff, Leonberg, Germany). The detection wavelength was set at 220 nm. Samples were introduced by manual injection using a variable-volume Rheodyne valve Model 7125 (Cotati, CA, USA) with a 20-μl and a 100-μl loop, respectively. Data acquisition was performed by a LC-CaDI 22-14 system using a McDAcq 32 program (supplied by Bischoff).

The scheme of integrated sample preparation for HPLC–electrospray ionization (ESI)-MS is shown in Fig. 2. The sample clean up–HPLC–ESI-MS experiments were performed on a fully automated LC-MS system from PE Sciex (Thornhill, Canada) addition-

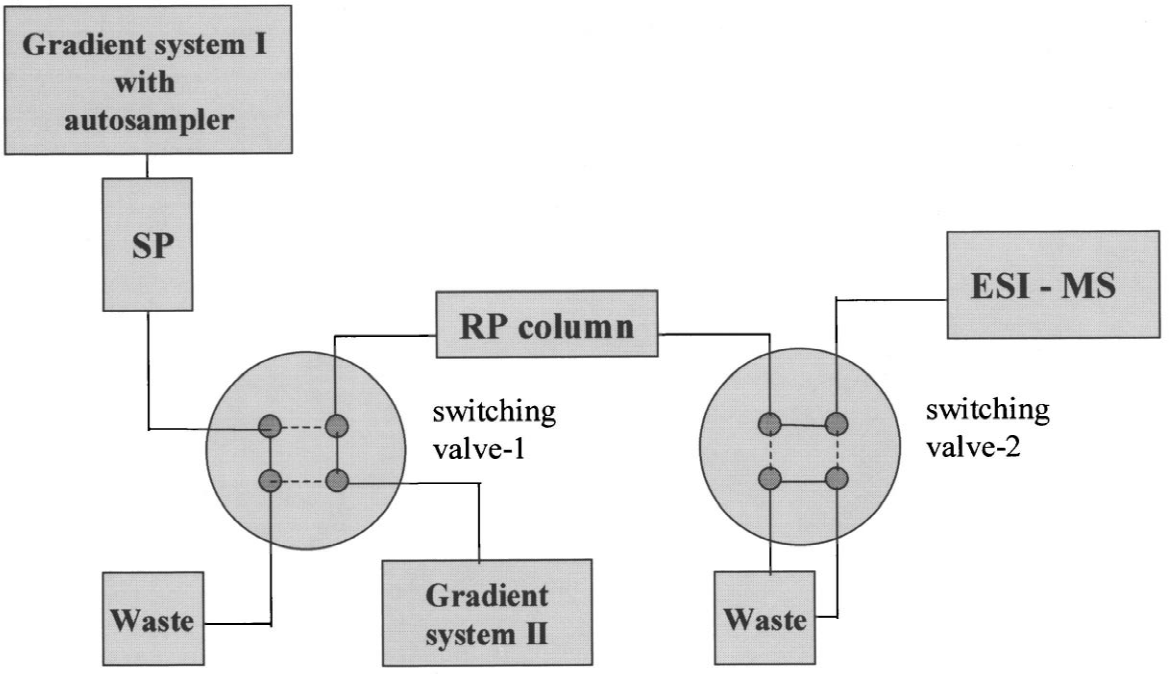

Fig. 2. Scheme of the set up used in the sample clean up–HPLC–ESI-MS system (—, position A; ----, position B).

ally equipped with two PE Sciex pumps and two pneumatically driven ten-port two position switching valves (Rheodyne). Control of the sample clean up–HPLC–ESI-MS system and data evaluation were achieved with the program ''Sample Control''. The data were reviewed in a programme ''Multiview 1.4''. Both software were supplied by PE Sciex and are typical for their mass spectrometers.

''Off-line'' experiments were carried out in our laboratory at the Johannes Gutenberg-University, Mainz, Germany, while the sample clean up–HPLC–ESI-MS experiments were carried out in the Department of Bioanalytical Chemistry at Astra Zeneca, Mölndal, Sweden.

Sample clean-up was performed using a silica based restricted access cation exchanger (supplied as a research sample by Merck) with the following properties: particle size d_p~5 μm, protein binding capacity approximately 0.2 g/g and a molecular mass exclusion limit for globular proteins of approximately 15 000.

For sample loading and rinsing of the matrix at the column, 10 m*M* phosphate buffer pH 3.5 containing 5 – 10% (v/v) acetonitrile (A) was used. The transfer eluent was a 10 m*M* phosphate buffer pH 3.5 with 1 *M* NaCl and 20% (v/v) acetonitrile (B). In the off-line sample clean-up studies we applied a gradient from 0 to 100% B in 10 min at a flow-rate of 1 ml/min for the elution of NPY and its fragments.

2.3. Reversed phase HPLC separation

The RP-HPLC separations were performed on a nonporous 1.5-μm silica C_{18} column Micra ODS II 33×4 mm I.D. (provided by Micra Scientific, Northbrooke, IL, USA). The elution of NPY and its fragments was achieved using a gradient from 20 to 60% (v/v) acetonitrile in water with 0.1% (v/v) formic acid and 0.01% (v/v) trifluoroacetic acid (TFA) in 4 min at a flow-rate of 0.75 ml/min. All experiments were carried out at ambient temperature.

2.4. Electrospray ionisation mass spectrometry

Detection was performed with a triple quadrupole mass spectrometer PE Sciex API 3000 equipped with a Turbo-Ionspray source. The LC flow-rate (0.75 ml/min) was split by means of a conventional T-piece to 100 μl/min. Scanning of first quadrupole source was done in the positive mode with a step size of 0.3 amu and a dwell time of 0.2 ms. The ESI voltage was kept at 4.5 kV. The scanning range was m/z 470–1100. The temperature of the turbo gas was 300°C.

3. Results and discussion

We introduced a new restricted access sulphonic acid cation exchanger based on a d_p=5 μm, 6 nm pore size spherical silica packed in a cartridge of 33×4 mm I.D. The restricted access sulphonic acid cation exchanger has a topochemically bimodal functionalized surface: the outer surface of the particles carries bonded diol groups attached via propoxy spacers; the inner surface, i.e., the pore walls, contains bonded sulphonic acid groups attached via carbonaceous spacers. The average pore diameter of the 5-μm spherical silica particles is approximately 6 nm, yielding a molecular weight exclusion limit for protonaceous matrix components of approximately 15 000.

When injecting a plasma sample on the RAMs column the matrix, constituents are excluded from the pore space and show a minimum adsorption at the external hydrophilic surface, whereas neuropeptide Y and its fragments can diffuse into the pores to interact with the pore surface. At the pH used during the sample application, all analytes were overall positively charged (compare Table 1). Accordingly, they are retained on the cationic surface sides of the precolumn. Since peptides generally have an N-terminal charge that will be neutralized at high pH values [31] we expect that cation exchange materials can even be used in the sample preparation of peptides that are not overall positively charged, i.e., peptides with lower p*I* values or when working with eluents with pH values up to 6.5. While the peptide analytes remain adsorbed to the RAMs column, the matrix will be eluted almost completely and will not be retained on the RAMs column during the washing step. By changing the eluent composition (i.e., increase the solvent strength) by enhancing the salt content the analytes are displaced from the RAMs column during the washing step and then transferred to the reversed-phase column for separation.

Injecting human plasma samples directly onto the RAMs column requires chromatographic conditions that prevent the precipitation of matrix proteins due to an inappropriate pH of the mobile phase or too a high percentage of organic solvent. The retention of neuropeptide Y and its fragments on the RAMs

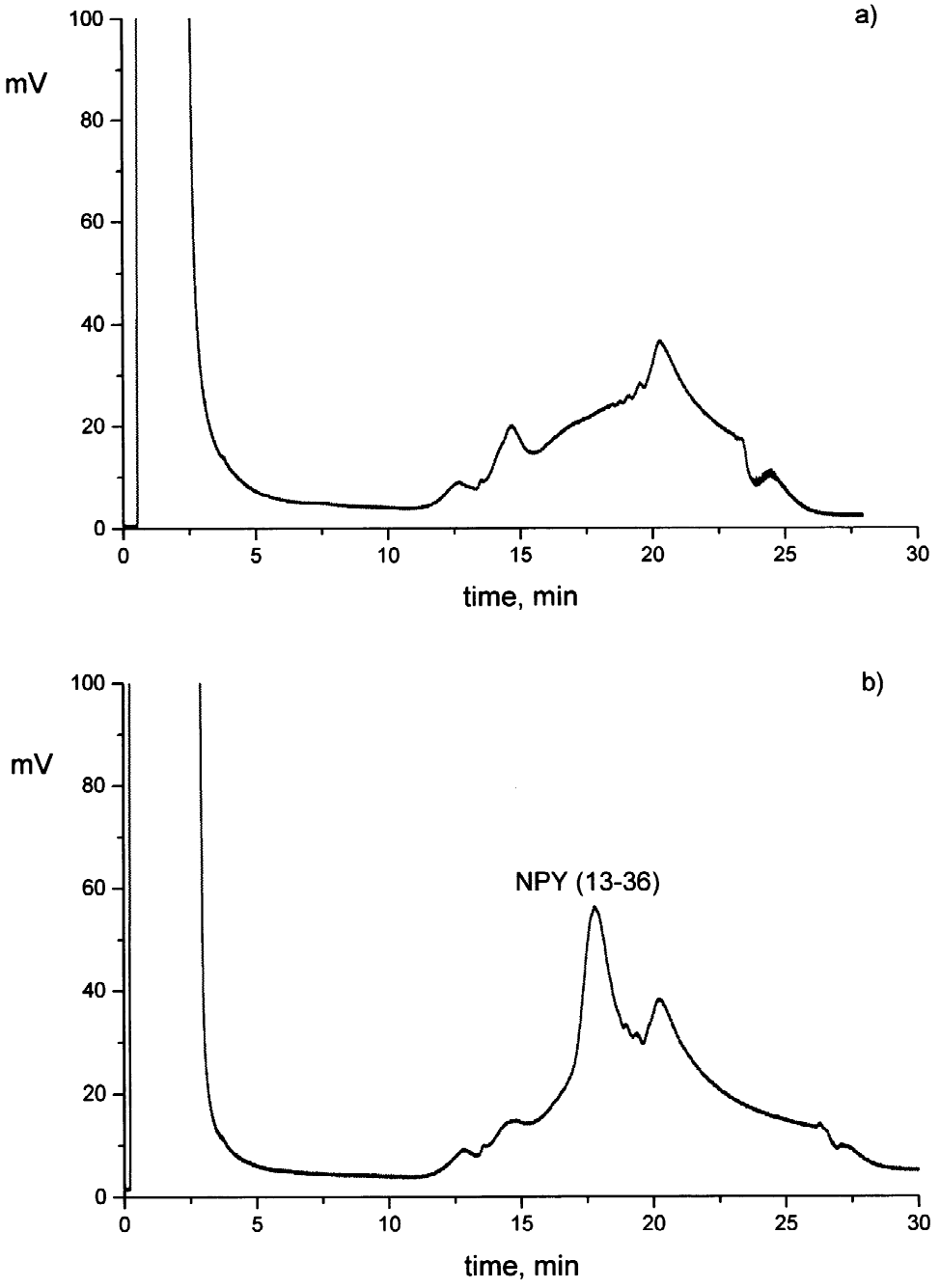

Fig. 3. Chromatograms of blank plasma (a) and plasma spiked with 20 μg of neuropeptide Y (13–36) (b), injection volume=100 μl.

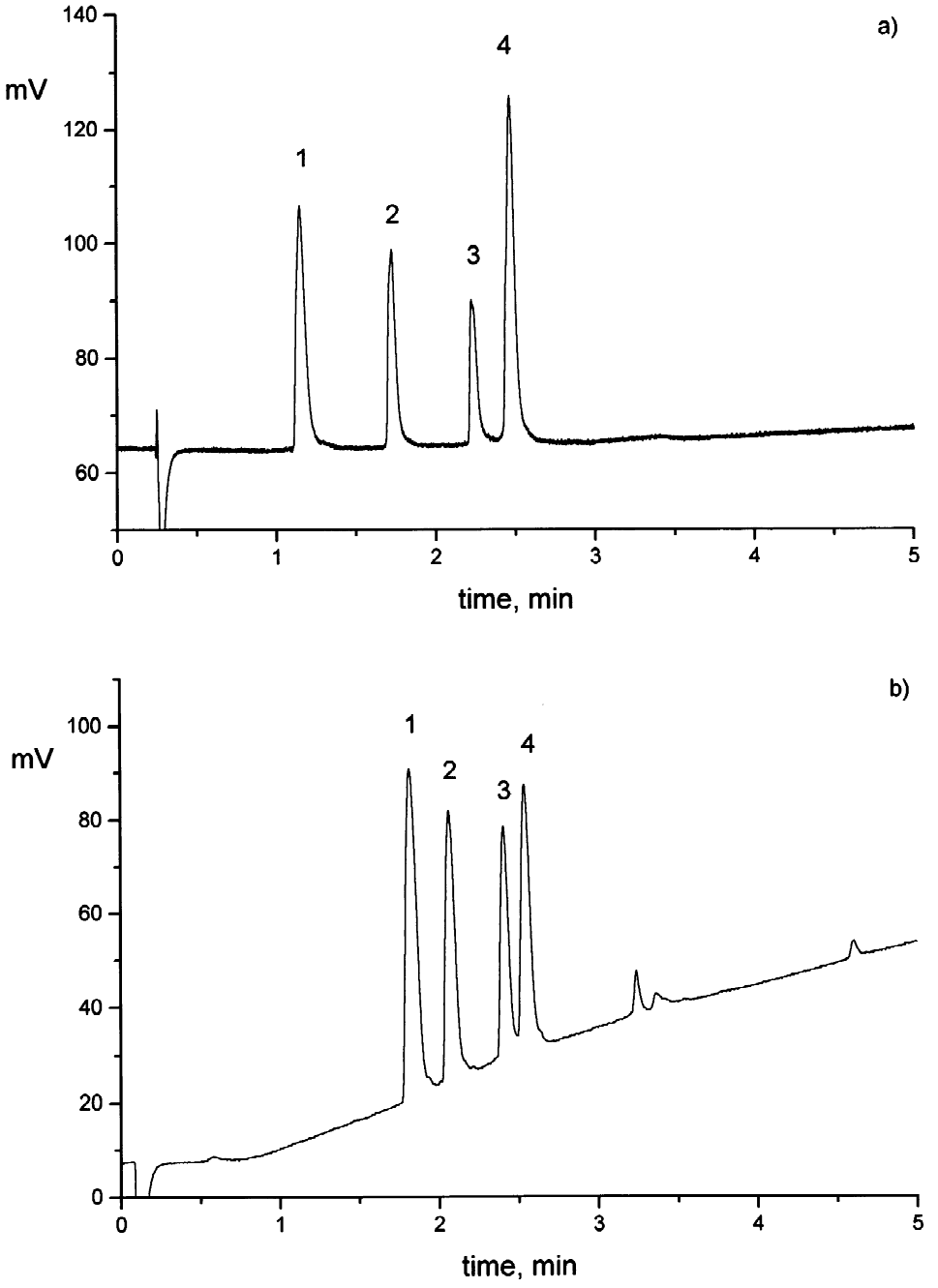

Fig. 4. Separation of a NPY mixture on a Micra ODS II (33×4.6 mm I.D.) column using 0.1% TFA (a) and 0.1% formic acid (b). 1=NPY (22–36), 2=NPY (18–36), 3=NPY (13–36), 4=NPY (3–36) and NPY (1–36). Injection volume=5 μl, conc=0.5 mg/ml, flow-rate=1 ml/min.

column was firstly examined using buffers at different pH values in the off-line mode. We found a mobile phase composition containing 10 m*M* phosphate buffer pH 3.5 with a maximum 10% (v/v) acetonitrile as optimal for the extraction of all targeted analytes. As an example, results for NPY 13–36 are shown in Fig. 3. A comparison was made between the chromatography of the blank (non-spiked plasma) (a) and the plasma spiked with 20-μg neuropeptide Y (13-36) samples (b). The peak due to the neuropeptide Y (13–36) was observed after the injection of the spiked plasma sample.

In order to establish the proper conditions for the sample clean-up step and the HPLC separation, experiments were carried out off-line, i.e., without the connection to the ESI-MS.

Fig. 4 illustrates the separation efficiency of neuropeptide Y and its fragments that can be achieved off-line on the non-porous 1.5-μm Micra NPS ODS column. The chromatogram demonstrates

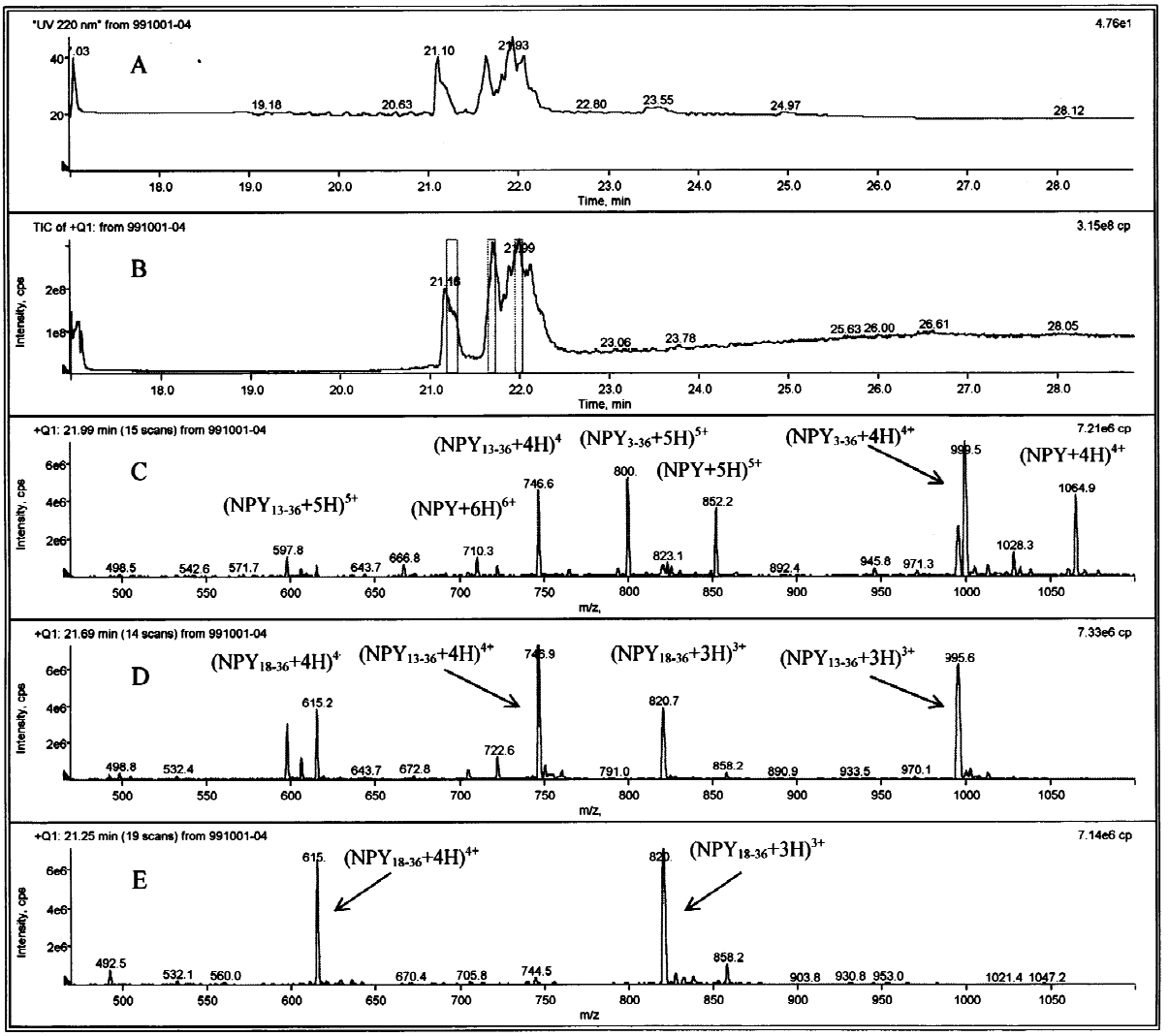

Fig. 5. Mass spectrum of NPY and its fragment mixture after separation on the integrated sample clean up–HPLC system. (A) UV trace; (B) TIC, spectrum at retention time; (C) 22 min; (D) 21.7 min; (E) 21.3 min.

that a fast separation of peptides is achieved using 0.1% TFA (a) as an additive and also 0.1% formic acid (b) as an additive. We later eliminated TFA to avoid ion suppression in ESI-MS and thus improve detection sensitivity.

It is difficult to separate NPY and NPY (3–36). In our set-up, separation is not crucial, since the analytes can be distinguished by means of MS detection. In contrast, immunoassays for NPY are in general cross-reactive with NPY (3–36) [32].

The final procedure developed was as follows:

The adsorption of neuropeptide Y and its fragments on the RAMs column was performed with a mobile phase of 10 m*M* phosphate buffer pH 3.5 with 5% (v/v) acetonitrile at a flow-rate of 1 ml/min at valve-1 position A. After 10 min, valve-1 was switched to position B and the concentrated analyte was transferred from the RAMs column to the Micra ODS column with 10 m*M* phosphate buffer pH 3.5 with 20% (v/v) acetonitrile and 1 *M* NaCl at a

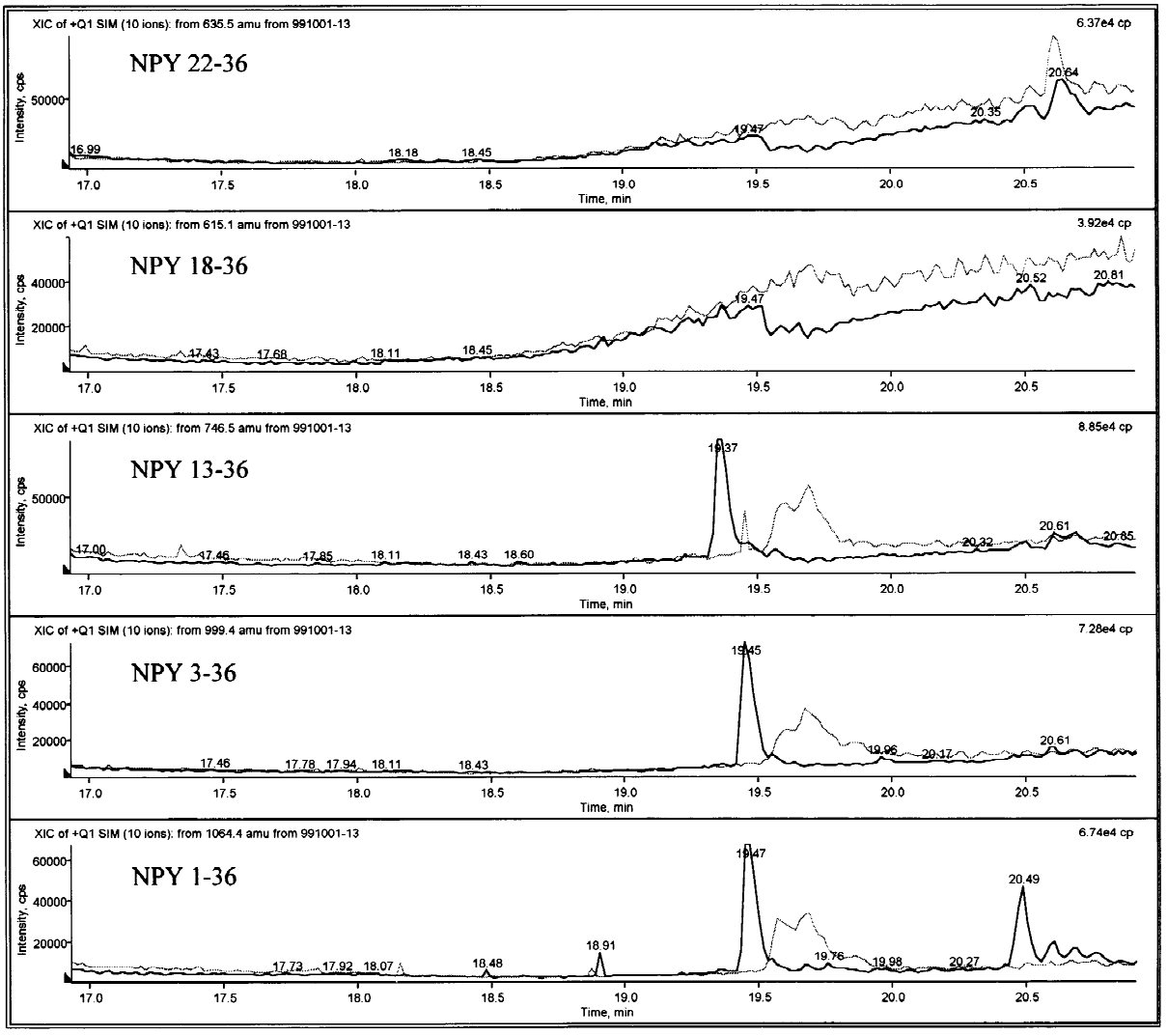

Fig. 6. Selected ion monitoring of a porcine plasma sample (black line) and pure water (gray line) after sample preparation and separation on a Micra ODS II column. The injection volume was 20 μl. Data were recorded in the SIM mode at m/z 635.5 (NPY 22–26), 615.1 (NPY 18–36), 746.5 (NPY 13–36), 999.4 (NPY 3–36) and 1064.4 (NPY).

flow-rate of 1 ml/min. From 15 to 17 min (valve-1 at position A, valve-2 at position B) the Micra ODS column was washed free from salt using a solution of 0.1% formic acid, 0.01% TFA and 5% (v/v) acetonitrile. In order to avoid contamination of the mass spectrometer, valve-2 was switched to position A from 17 to 21 min during the separation of the peptides and their fragments on the Micra ODS column. The separation of the peptides and their fragments was performed by a linear gradient from 20 to 60% (v/v) acetonitrile in water with 0.1% formic acid and 0.01% TFA in 4 min at a flow-rate of 0.75 ml/min.

Compared to the traditional sample clean-up and the HPLC separation with a 3–5 μm silanized silica reversed-phase column, the use of the RAMs and the 1.5-μm non-porous Micra NPS ODS column lead to a drastic reduction in the sample preparation and analysis time to about 20 min.

The system was examined with samples containing a mixture of the peptide fragments. A 100-μl aliquot of peptide solution (concentration: 200 μg/ml) was injected into the system and a mass spectrum was obtained. The following fragments were identified: NPY (1–36), NPY (3–36), NPY (13–36) and NPY (18–36) as shown in Fig. 5. The limit of detection of NPY was 5 nmol/ml with an injection volume of 20 μl (data not shown).

The porcine plasma samples were also examined on the sample clean up–RP-HPLC–ESI-MS system. We injected volumes of between 20 and 100 μl of plasma samples. Even with a 20-μl injection the neuropeptide Y with its fragments could be detected. Fig. 6 shows the ion-chromatogram obtained after a 20-μl plasma injection. The data were recorded in the SIM (selected ion monitoring) mode at m/z 635.5, 615.1, 746.5, 999.4 and 1064.4. Three NPY fragments (NPY 1–36, NPY 3–36 and NPY 13–36) were detected. To be sure that we were not observing peptides from carryover we also compared chromatograms of a porcine plasma sample and pure water at an injection volume of 20 μl. No carryover was observed.

4. Conclusion

We have demonstrated that silica based restricted access sulphonic acid cation exchange columns can be successfully applied to the sample clean up of neuropeptides in porcine plasma and can be integrated into a fully automated system for the analysis by reversed-phase-HPLC–ESI-MS. The procedure has to be validated and tested with regard to its robustness using real samples.

Further investigations are directed towards the enhancement of the sensitivity of the method by applying RAMs columns and Micra ODS columns with 2-mm I.D. With the reduction of the column I.D. we expect to improve the limit of detection by approximately a factor of 100 and more. The miniaturization of the reversed-phase column would allow the direct coupling to the MS without a split before the ESI-MS instrument. The sample throughput could be increased and analysis time reduced by installing an additional switching valve and a second RAMs column. Further improvement to selectivity of the system is in progress.

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