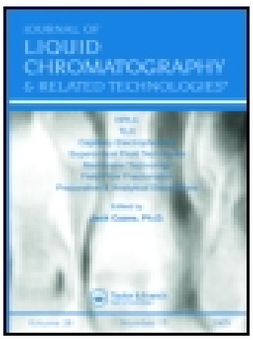

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Automated SPE and nanoLC–MS analysis of somatostatin

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ABSTRACT

Plasma peptides are widely used in clinical diagnosis and therapy monitoring. Our aim was an efficient system development for the enrichment of small, low abundant peptides from plasma by robotized sample preparation. The automation of SPE yields additional time saving and improves system robustness and repeatability. Automation is based on combining a Waters SPE kit with Oasis HLB sorbent and a multichannel liquid handling workstation with cheap commercially available electronic devices such as a programmable logic controller (PLC) and an AVR controller. Reversed phase nano liquid chromatography coupled with a sensitive quadrupole time of flight mass spectrometer (QTOF MS) and it was used for the quantitative determination of somatostatin (SST). We quantified SST from mouse plasma, where lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were 2.5 and 8.3 fmol/ml, respectively. We investigated linearity of response, accuracy, precision, recovery, reproducibility and stability of SST during both short-term sample processing and long-term storage. This method allows reliable quantification of plasma peptides. The developed automated sample preparation solid phase extraction method can be easily and conveniently adopted for different volumes and amounts of sample in routine analysis using controlled vacuum. The highest advantage is enhanced reproducibility, which makes it suitable for the investigations of large sample cohorts in clinical studies.

KEYWORDS

Automation; controlled vacuum; liquid handling robot; nanoLC–MS; somatostatin; SPE/LC

GRAPHICAL ABSTRACT

Somatostatin analysis by automated SPE and nanoLC-MS.system. Biologically important compounds can be examined from plasma and the system is suitable for the investigations of large sample cohorts in clinical studies.

Introduction

The neuropeptide somatostatin (SST) is a cyclic tetradecapeptide, which was first isolated from sheep hypothalamic cells. SST exists in two main forms: quantitatively predominant, but less bioactive SST-14 (consisting of 14 amino acids) and more bioactive SST-28 (NH_2-terminally extended 28 amino acid form).[1] SST is found in a wide range of tissues including the central nervous system and peripheral tissues.[4] SST is known to play an important role in the hypothalamic regulation of growth hormone secretion and inhibits the release of thyrotropin releasing hormone, dopamine and norepinephrine. It is a potential mediator of antidepressant effects.[25] In the peripheral nervous system SST has inhibitory effects on nociception and inflammatory processes.[27] SST exerts a number of effects on neuroendocrine, cognitive behavioral, autonomic and visceral functions.[8] Disorders in

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SST metabolism have been proposed to contribute to various psychiatric and neurological disorders SST concentrations in general are correlated with neurological and psychiatric disease states. Levels of SST are consistently decreased in the cerebrospinal fluid of patients with Alzheimer's disease and frontotemporal dementia. Reductions have also been reported in patients with multiple sclerosis or depression.[2,5,11,19,20]

Somatostatin-14 has a very short serum and plasma half-life (2–3 min) and due to this the therapeutic use of it is limited, but analogues of the hormone are used for certain diseases. During laboratory procedures this time can be prolonged using inhibitors and keeping samples at cold temperature. That decreases the susceptibility of this compound to endogenous proteases.[21]

Mass spectrometry (MS) has become an important tool in the study of neuropeptides through the method's ability to directly detect ions at a given mass-to-charge (m/z) ratio, even in complex biological mixtures (for example plasma, urine, cerebrospinal fluid, saliva etc.). The fastest way to determine the identity of peptide fragments produced by neuropeptide-processing, -converting, or inactivating of enzymes is to use matrix-assisted laser desorption ionization (MALDI) MS. SST-14 due to its high concentration in the tissue can be measured from brain sections directly with MALDI–time-of-flight (TOF) MS.[28] Quantification of peptides in general can be achieved by several techniques. Liquid chromatographic separation followed predominantly by electrospray ionization MS represents a sensitive and robust technique, but radioimmunoassay (RIA)[21,26] and chromatography with UV detection are also in use. Identification of SSTs has already been published with LC–MS from hypothalamic samples, where the authors used *in situ* hybridization to follow changes in the gene expression.[25]

Solid phase extraction (SPE) is a valuable and widely used technique for the purification and selective extraction of complex biological samples (plasma, urine etc.). The use of a 96-well SPE block can save time and is cost-effective. The automation of SPE yields additional time saving and improves system robustness and repeatability. Generally, automated SPE systems are applied in investigations of small molecules, in pharmacokinetics screening studies for example VLA-4 antagonists,[29] doramectin[14] and neurotransmitters from blood,[17] toxicology monitoring for example tetrahydrocannabinol and metabolites from urine.[16] Automated peptide and protein analysis requires more complicated methods.[3,6,17]

Our primary aim was the quantitative determination of the neuropeptide from blood samples. Blood samples contain significantly lower concentration of SST-14 than hypothalamic samples therefore we used reversed phase nano liquid chromatography coupled with a sensitive quadrupole TOF MS. We aimed to develop a method ideal for clinical laboratories. Therefore, we automated the complete sample pretreatment to make it quick, precise, highly repeatable and fit for the analysis of large number of samples.

Materials and methods

Chemicals and reagents

The SST-14 standard and trifluoro-acetic acid were purchased from Fluka Chemie AG (Buchs, Switzerland). Solvents, ultrapure water, methanol and acetonitrile (Hipersolv, Chromanorm LC–MS grade) were purchased from VWR (Radnor, PA, USA). LC–MS grade CHROMASOLV formic acid, acetic acid, ammonium-hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amicon Ultra 10 MWCO centrifugal filter devices (CFD) were from Merck-Millipore (Darmstadt, Germany). The SPE kit with Oasis HLB 96 well plates was obtained from Waters (Milford, MA, United States).

Animals and sample pretreatment

Bagg Albino (20–25 g) mouse strain (BALB/c) were obtained from Charles River, Hungary and bred in the Animal Centre of the University of Pécs under standard pathogen-free conditions.

Animals were fed and watered ad libitum under light/dark cycles of 12/12 hr. All procedures were performed in accordance with the ethical guidelines and under approved protocols (ethical permission number: University of Pecs BA02/2000-15024/2011).

Blood samples were collected by cardiac puncture under ketamine (100 mg/kg i.p., Richter, Hungary) and xylazine (5 mg/kg i.p., Lavet, Hungary) anesthesia. The animals were fasted overnight before sampling to avoid gastrointestinal SST-14 release. The cooled Protein Lobind tubes (Eppendorf, Wien, Austria) contained 40 µl protease inhibitor (EDTA) and 20 µl peptidase inhibitor cocktail (Sigma-Aldrich, Hungary) and aprotinin (Trasylol, Bayer, Germany). Blood samples were centrifuged at 1,000 rpm for 5 min and 10,000 rpm for 10 min at 4°C to separate plasma. Plasma were stored at −20°C until further processing. During the total sample collection and preparation LoRetention tips (Eppendorf, Wien, Austria) were used to avoid protein lost. Six mice were used in the animal study.

Instrumentation

Extraction was achieved by an eight channel Hamilton Microlab Starlet liquid handling workstation (Hamilton Co., Bonaduz, GR, Switzerland). This robot was controlled by a PLC (Siemens S7 300) and an AVR controller (Amtel atmega8). We connected a membrane vacuum pump (GAS DOA 504) to the extraction system and an SMC vacuum controller valve (SMC ITV 009).

Control of protein removal was performed with an Autoflex II TOF/TOF MALDI–MS instrument (Bruker, Bremen, Germany).

For the Analysis of SST-14 we used an EASY II nanoLC—Maxis 4G UHR-QTOF MS (Bruker, Bremen, Germany) system.

Automation

The complete sample preparation has been automated. 1 ml water was used for washing, than the phase was activated with 1 ml mixture of methanol/water (v/v, 1/1). 200 µl 0.1% TFA (in water) was added to 800 µl sample and it was loaded to the phase. 1 ml methanol/water/ammonium-hydroxide

(v/v/v, 5/93/2) and 1 ml methanol/water/acetic acid (v/v/v, 5/93/2) solutions were used alternately to wash the plate twice, respectively. The extracted components were eluted from the phase with 500 µl of methanol/water/formic acid (v/v/v, 65/33/2).

The developed method and extraction was integrated to the automated system, which consisted of the liquid handling workstation and an external SPE system. To spare time and to enhance reproducibility we integrated the SPE kit—which was designed for manual use—into the liquid handling workstation. The physical dimensions of the vacuum manifold of the Waters SPE-kit (175 mm × 125 mm) allowed us to fix it on the motherboard of the workstation. We connected the membrane vacuum pump and the PLC to control it. We designed and set up an AVR controller between the software of the workstation (Hamilton Venus two 4.3) and the PLC to ensure online communication. We applied impulse modulated communication through a plugin type assembly program directly between the USB port of the computer and the digital input card of the PLC (Figure 1) as a trigger. We covered the unused parts of the Oasis 10 mg HLB 96-well plate cartridges with a polyethylene foil as we had to maintain the vacuum, while the full capacity of the well plate was not used. On SPE cartridges smooth flow parameters must continuously be ensured to get good reproducibility. Due to the differing number and the uneven viscosities of the samples vacuum fluctuations could occur. To compensate these we built in a feedback type vacuum control system. The vacuum measurer built in the manifold is directly connected to the PLC which uses a proportional-integral-derivative algorithm and a vacuum controller valve to compensate quickly and precisely the deviation from the value of reference signal. A flexible controller program was written for the robot which supports different numbers and types of samples without any change in the script. The program uses selection, sequence, iteration, local and global variable declaration supported by the Hamilton Venus two software and it was provided by a graphic panel. That way we have a complete sample pretreatment system, which can easily be used by any operator including end users without programming experience.

After SPE, sample eluates were loaded in CFD and centrifuged at 10,000 rpm for 30 min to remove proteins and particles. Filtrates were tested by MALDI TOF/TOF and if protein was found the filtration had been repeated. The protein free filtrates were loaded into autosampler vials and nanoLC–MS was carried out (Figure 2).

MALDI-TOF control of effectiveness of protein excluding

Despite of the cautious pretreatment protein residues cannot always removed perfectly. We applied quick MALDI–TOF measurements to test our samples of any possible remaining proteins (Figure 2),[22] which could later be precipitated and cause column blockage. To carry out these controlling experiments an Autoflex II MALDI–TOF/TOF MS (Bruker Daltonics, Bremen, Germany) was used. The system was controlled by Flexcontrol 3.4. Data was evaluated using Flexanalysis 3.4. The centrifuged samples were enriched on a Protein Anchor chip target plate (MTPAnchorChip™384 T F, Bruker Daltonics, Bremen, Germany) by using 1 µl of sample solution. After that 1 µl freshly prepared diluted (0.7 mg/ml) α -cyano-4-hydroxycinnamic acid in acetonitrile/0.1% TFA (1/2, v/v) solution was spotted onto the samples. The mass spectrometer was equipped with a 1 kHz Smart beam II solid-state Nd:YAG UV laser (Lasertechnik Berlin GmbH, Berlin, Germany). The instrument was operated in linear mode. The accelerating voltage was set to 20.00 kV. External calibration was performed using Bruker Protein II Calibration Standard (#207234, Bruker Daltonics). Protein masses were acquired in the mass range of 5–80 kDa.

NanoLC–MS

Separation was performed by Bruker Easy nanoLC II (Bruker Daltonics, Bremen, Germany) using a 100 mm × 75 µm

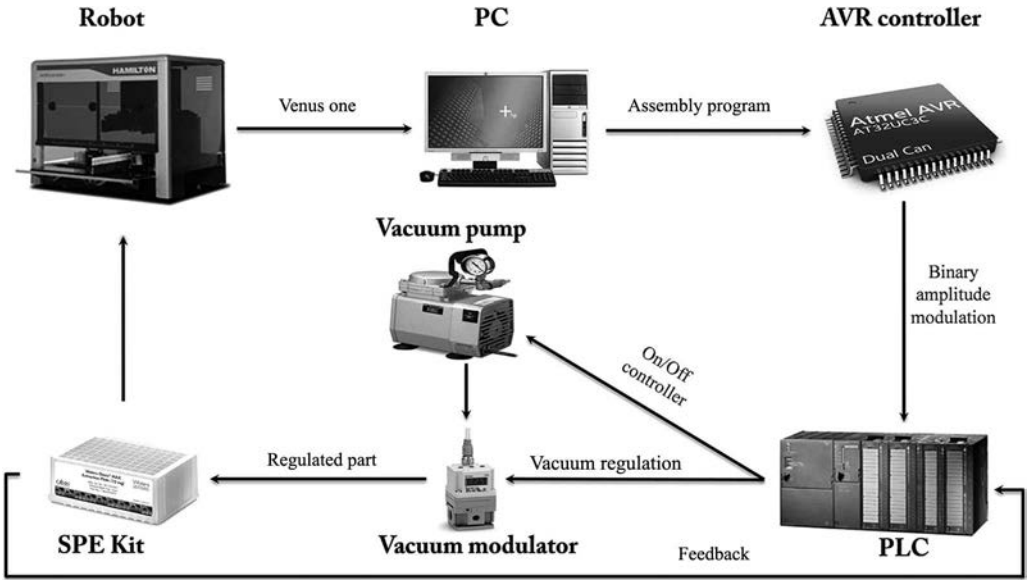

Figure 1. Scheme illustration of automated SPE sample preparation system.

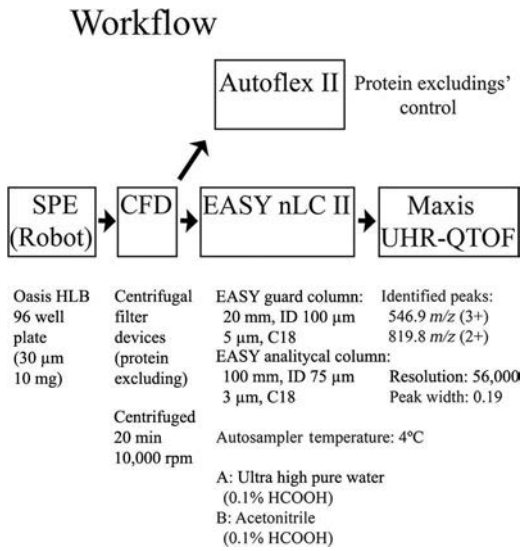

Figure 2. Schematic experimental workflow.

(3 µm) C18 Thermo Easy-Column (Unicam Kft, Budapest, Hungary) and a 20 mm × 100 µm (5 µm) C18 pre-column at 23°C. The injected volume was 1 µl for each analysis. The autosampler was set to 4°C. A binary gradient consisting of mobile phases A and B (A: water-0.1% formic acid, B: acetonitrile-0.1% formic acid,) was applied. The gradient profile was 10–30% B in 2 min, than 30–80% in 28 min. The column was washed with 90% B for 6.5 min and equilibrated to the initial conditions with a 1 min linear gradient and an isocratic period of 6.5 min.

The mass analyzer was a Bruker Maxis 4G UHR-QTOF MS instrument (Bruker Daltonics, Bremen, Germany) coupled with a nano-ESI source. The instrument was controlled by the software Compass 1.3 (Bruker Daltonics, Bremen, Germany). The mass spectrometer was operated in positive mode. The scanning mass-to-charge range was m/z 100–3,000 at 1 Hz acquisition rate. Nitrogen was used as nebulizer gas pressure was set to 0.6 bar, drying gas flow was 7 l/hr at 180°C and the capillary voltage was set to 3.8 kV. The quantification and data evaluation were performed by the Data Analysis v.3.4 software (Bruker Daltonics, Bremen, Germany), peak picking was achieved by applying Apex algorithm on extracted ion chromatograms (EIC).

Results and discussion

SST-14 determination by nanoLC–MS

Earlier, we used a specific sensitive RIA method for the measurement of SST-like immunoreactivity in human plasma and other biological samples.[24] This analytical technique requires minimum 3 ml plasma. Since only 1 ml blood can be obtained from a mouse, this method was not optimal in the present animal experiment. The adoption of MS-based methodologies for peptide analysis has been relatively slow. This is in part due to the difficulty of achieving the same level of sensitivity of immunological methods.[18] The main cause of low sensitivity is that the peptide ion current is divided amongst the multiple charge states that are commonly observed in electrospray ionization.[18] Summation of different transitions can compensate to some extent the loss in signal intensity inherent to the appearance of multiple charge states.[10] The combination of an effective sample preparation method and the use of up-to-date instruments can enable peptides to be detected with sensitivities similar to immunological-based approaches.[18]

Oasis HLB extraction products, which was used to SPE, contain a resin made from a co-polymer of divinybenzene and vinyl pyrrolidinone. The pyrrolidinone functionality acts as an imbedded hydrophilic group thereby provides wetting properties. The pyrrolidinone functionality also provides enhanced retention for some polar analytes. The divinybenzene, as liphophilic monomer provides reversed-phase property for analyte retention. It was used to peptide desalting and separation but the number of shared peptides only 45% were same compared to reversed-phase C18. That can be explained with the completely different chemistry of the stationary phases.[12]

Reversed-phase LC is an important tool for peptide separation. In contrast to the well-understood chromatographic behavior of small molecules, the behaviour of peptides under different chromatographic conditions are more complicate.[30]

For example, as expected, mobile phase related factors had a significant influence on the retention for most peptides as well as on MS sensitivity. TFA as ion-pairing reagent in the mobile phase was clearly very detrimental to peak area compared to formic acid. TFA is a strong and rather hydrophobic acid that forms stable ion-pairs with positively charged amino acid residues, enhancing peptide retention on the column. On the other hand, formic acid is a weaker acid that forms more labile ion-pairs, which is beneficial to analyte ionization in the MS source, but is less favourable to peptide retention on the stationary phase.[15] Furthermore, the retention behaviour of the peptides with different charge are variable and the relative peak areas are affected by ionization efficiency of peptides in different TFA conditions.[13] Thus, TFA, as ion-pairing reagent was not use for the analytical separation, only in the automated SPE processes.

Two SST-14 ions were observed by nanoLC–MS, doubly charged (819.8 m/z) and triply charged (546.9 m/z). The triply charged ion was more intense and the ratio of the two SST-14 ions was constant (3.62 ± 0.02). The peptide sequence was confirmed by MSMS. Similarly to Dillen,[9] the acceptable fragmentation was only observed for the triply charged ion. The doubly charged peptide breaks only down to fragments at high collision energy and the obtained fragment ions have lower intensity compared to fragment ions obtained from the triply charged peptide.[9] Therefore, quantification was based on the cumulative peak areas of the triply charged (546.9 ± 0.05 m/z) and doubly charged ion (819.8 ± 0.05 m/z).

NanoLC–MS method validation

Our sensitive nanoLC–MS method enabled us the determination of SST-14 from mouse plasma containing protease and peptidase inhibitors. Validation was also performed in plasma. We investigated linearity of response, accuracy, precision, recovery, reproducibility and stability of SST-14 during

both short-term sample processing and long-term storage. The linearity of the method was determined by a weighted least square regression analysis of standard plot associated with a five-point standard curve. Analytical curve was found to be linear in the range of 0.16–900 fmol/ml. Correlation coefficients (R^2) were consistently greater than 0.995 during the course of validation. The LLOD (3S/N) and the LLOQ (10S/N) were 2.5 and 8.3 fmol/ml; they were calculated from EICs of 10 fmol/ml standard solution. These detection limits are similar to earlier described,[9] where 3 pmol/ml (5 ng/ml) SST was determined by high-resolution TOF MS and 600 fmol/ml (1 ng/ml) by triple quadrupole MS as LLOQ. Reproducibility was summarized in Table 1. The deviation of intra- and inter-day precisions were ≤9.7%, whereas intra- and inter-day accuracies were in the range of 92.3–108.9%. These results indicate that the method's precision and accuracy were adequate for the level of analyte concentration in the samples. The applied nanoLC runtime was relatively long (44 min), however, using a column selector and an extra pump the runtime could be decreased.

Automated assay validation

With the PLC instrument we were able to interconnect the liquid handling workstation; the SPE kits and the external vacuum pump online (Figure 1). The developed PLC method contained 384 commands. Our online system is flexible; we can easily adjust it to different volumes (100–1,500 µl) and different number of samples (1–96). Online sample preparation had to be interrupted only for protein removal, which was achieved on CFDs (Figure 2). Processing of a full loaded plate (96 samples) required less than half an hour. With this automated SPE system using proper solid phase and eluting program different components of plasma (proteins, peptides, lipids, metabolites etc.) can be extracted.

During method development we investigated reproducibility, recovery and various stability indicators of the Waters stationary phase by nanoLC–MS. For that purpose, plasma samples were used, which were stored at room temperature for 24 hr without inhibitors (protease inhibitor EDTA, peptidase inhibitor cocktail, aprotinin). This storage resulted in SST-14-free (<LLOD, data not found) plasma samples due to degradation. They were spiked with fresh SST-14 standards to the final concentration of 1, 10, 100 fmol/ml and they were added the inhibitor cocktail. SST-14 could be identified with nanoLC–MS using automated SPE system

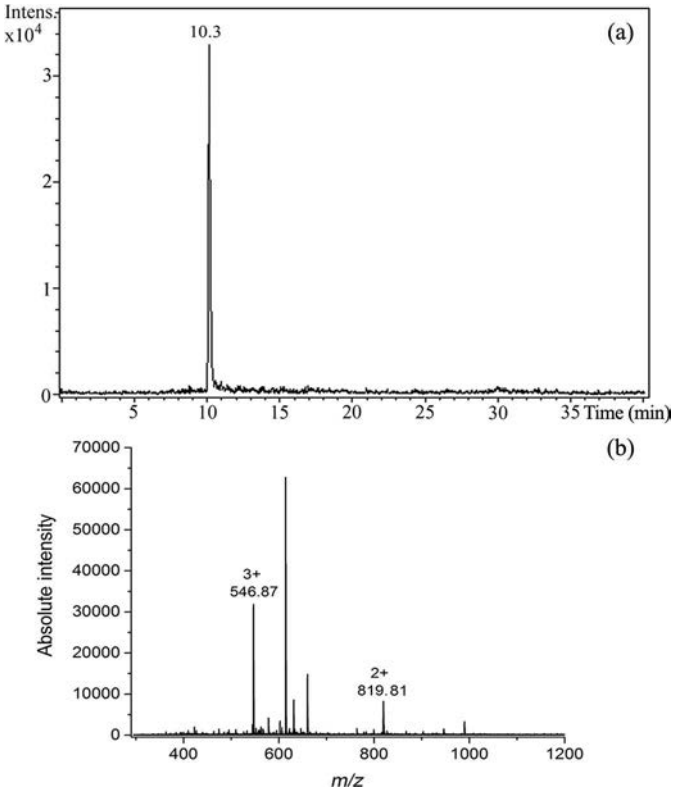

Figure 3. Analysis of the mouse sample by SPE-nanoLC–MS system. Panel a: EIC of triply charged somatostatin (*m/z* 546.9), b: mass spectrum of the peak at 10.3 min, *m/z* 819.8 is the doubly charged somatostatin, *m/z* 546.9 is triply charged somatostatin. The calculated SST-14 concentration was 24.9 fmol/ml.

(Figure 3). Spiked plasma samples were used for recovery experiments. Recoveries (±SD) were found at the three different concentration levels (1, 10, and 100 fmol/mL), and found to be 73.8% (±9.9), 88.28% (±4.5), and 79.8% (±6.0), respectively. These results indicate that the automatic SPE efficiency for SST was satisfactory, and consistent in the examined concentration range.

Samples were at room temperature only during preparation and SPE; they were kept at 4°C in the autosampler of the nanoLC otherwise at −80°C. The stability of the SST-14 spiked samples were investigated at two concentration levels (10 and 100 fmol/ml) to cover expected conditions during analysis, storage, and sample processing, which include the stability data from various stability exercises like bench-top, freeze/thaw, processed sample, and long-term stability tests

Table 1. Intra-day and inter-day precision and accuracy for the analysis of SST-14 in standard solution (calculated from data from six separate runs), $n = 6$.

Nominal concentration (fmol/ml)	Measured concentration (fmol/ml ± SD)	Precision (CV, %)	Accuracy (recovery, %)
Intra-day variation			
1	0.96 ± 0.08	8.3	96.1
10	10.89 ± 0.92	8.5	108.9
100	97.52 ± 3.52	3.6	97.5
Inter-day variation			
1	0.92 ± 0.07	7.6	92.3
10	10.55 ± 1.02	9.7	105.5
100	99.06 ± 4.56	4.6	99.1

Table 2. Precision and accuracy for the analysis of SST-14 in spiked plasma (calculated from data from six separate sample) were measured at three different stability conditions, $n = 6$.

Nominal concentration (fmol/ml)	Measured concentration (fmol/ml)	Precision (CV, %)	Accuracy (recovery, %)
Bench top stability			
10	9.01 ± 0.99	11.0	90.1
100	93.62 ± 5.70	6.0	93.6
Processed sample			
10	9.34 ± 0.87	9.3	93.4
100	95.93 ± 5.65	5.9	95.9
Freeze-thaw			
10	8.87 ± 0.95	10.7	88.7
100	91.06 ± 4.94	5.4	91.1

Table 3. Plasma somatostatin concentrations of mice, $n = 6$.

ID	Measured concentration (fmol/ml ± SD)
1	24.9 ± 1.2
2	23.9 ± 0.3
3	23.2 ± 0.6
4	24.9 ± 0.1
5	25.9 ± 1.0
6	21.9 ± 0.6
Average	24.1 ± 2.2

(Table 2). The stability results indicate that SST-14 spiked mouse plasma samples were stable for at least 6.0 hr at room temperature, for at least 48 hr in final extract at 15°C when stored in the autosampler and during three freeze-thaw cycles when stored at around −80°C and thawed to room temperature.

Animal study

To prove biological applicability we analyzed six mouse plasma samples, which showed little differences in the concentration of SST-14. The average concentration was 24.1 ± 2.21 fmol/ml (Table 3). This value is approximately the half of the concentration found in previous study.[7] The reason of finding lower concentration of SST-14 compared to literature data probably comes from cross reactivity of radio-immunoassay technics, which causes an increase in the signal.[23]

Conclusions

A reliable, selective and specific analytical method for the determination of SST-14 in plasma samples has been developed and validated. Samples were prepared by automatic SPE, separated on a C18 column and quantified by nanoLC–MS. This method exhibited adequate sensitivity, acceptable precision and excellent stability for the quantification of SST-14 in mouse plasma containing protease and peptidase inhibitors.

Automation in general can increase the sample throughput and also increases repeatability, stability, precision, and accuracy. The added values of our system are the integration of the SPE into the liquid handling robot and the use of controlled vacuum. Controlled vacuum plays a very important role in the reproducibility of SPE. In future, the method could be applied *in vivo* and clinical tests with large sample cohorts.

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