Journal of Chromatography B, 879 (2011) 2501–2506

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Capillary electrophoresis coupled with mass spectrometry for the evaluation of substance P enzymatic degradation by SaOS-2 human osteosarcoma

Antonella Cavazza[a,*], Claudio Corradini[a], Mario Marini[b], Luigi Giorgio Roda[b], Angela Valenti[c]

[a] Dipartimento di Chimica Generale ed Inorganica, Chimica Analitica, Chimica Fisica, Università degli Studi di Parma, Parco Area delle Scienze, 43124 Parma, Italy
[b] Dipartimento di Neuroscienze, Università degli Studi di Roma "Tor Vergata", via Montpellier 1, 00133 Roma, Italy
[c] Dipartimento di Chirurgia, Università degli Studi di Roma "Tor Vergata", via Montpellier 1, 00133 Roma, Italy

ARTICLE INFO

Article history:
Received 31 January 2011
Accepted 30 June 2011
Available online 6 July 2011

Keywords:
Substance P
Capillary electrophoresis
Mass spectrometry
Free amino acids
Peptidases

ABSTRACT

A new analytical method for the detection and the quantitative evaluation of the undecapeptide substance P by capillary electrophoresis coupled with ion trap mass spectrometry (CE–MS) by a co-axial sheath liquid interface has been developed. Conditions of analysis employed an acidic buffer and a 60 cm fused silica capillary installed by overcoming the UV window position, thus allowing to perform the analysis in a brief time. The method has been applied to the evaluation of substance P enzymatic hydrolysis during incubation with the human osteosarcoma SaOS-2 cell line. The analysis of amino acids derived from the cleavage of substance P has been also carried out simultaneously under the same electrophoretic conditions allowing the description of a kinetic of amino acid formation, parallel with substance P disappearance. The amounts of intact substance P and of free amino acids were monitored along 600 s of incubation time. A steady decrease of substance P as function of reaction time was observed. Peptide's half-life was found to be about 4.3 s, indicating an extremely fast hydrolysis in the presence of the SaOS-2 cells. Proline, phenilalanine and methionine were the predominant free amino acids recorded. Obtained results lead to hypothesize the occurrence of endopeptidases activity, followed by aminopeptidases responsible for the release of free amino acids originated after primary bond cleavage.

1. Introduction

Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 [1]) is an undecapeptide present in body districts such as nervous system, gut, plasma [2], and also in bone [3]. This peptide is involved in many physiological processes [4]: specifically, it is reported to participate to inflammatory phenomena [5], including those associated with bone reshaping [6], and with rheumatoid arthritis [7]. As for other signal peptides, the local concentration of substance P must be lowered to base levels to terminate its activity. However, the very low accessibility of bone tissue may hamper inactivation processes such as diffusion, re-uptake, and especially hydrolysis by plasma enzymes (e.g., [8]). If these considerations are correct, hydrolysis by bone cell-expressed enzymes may represent the primary inactivation mechanism for bone-active substance P. Consequently, knowledge of these processes could help to clarify the role of this peptide in bone morphogenesis under both physiological and pathological conditions [6,9,10].

Both substance P terminals are resistant to exopeptidases: the amidated C-terminal Met prevents carboxypeptidase activity, while the Pro in position 2, with the exception of aminopeptidase P [11], hinders the activity of aminopeptidases [12]. Consequently, substance P degradation is catalyzed by endopeptidases: the Pro2-Lys3 bond by dipeptidylpeptidase IV (DAP IV, EC 3.4.14.5 [8,13]), the Gln6-Phe7, Phe7-Phe8 and Gly9-Leu10 bonds by neutral endopeptidase (NEP, EC 3.4.24.11 [13–15]), the Phe8-Gly9 and Gly9-Leu10 bonds by angiotensin converting enzyme (ACE, EC 3.4.15.1 [14]) and the Pro4-Gln5 by post-proline cleaving enzyme [16]. The ineffectiveness of exopeptidases implies that free amino acids can only be released by secondary degradation, by either endopeptidase or exopeptidase, of fragments released by the activity of endopeptidases. On the other hand, substance P's secondary hydrolysis appears to be functionally significant, because the activity is retained not only by the C-terminal [17] but also by the N-terminal [18] fragments. For studying these phenomena, we developed an analytical method for quantitative detection of substance P, and of the free amino acids possibly released as its hydrolysis by-products by capillary electrophoresis coupled with mass spectrometry.

Capillary electrophoresis coupled with mass spectrometry is a relatively new technique which has been used lately and widely in many applications of analytical science concerning food analysis [19,20], and biological and pharmaceutical topics [21,22]. Its suitability for protein and peptide analysis in biomedical applications has been shown in a recent review concerning clinical proteomics

* Corresponding author. Tel.: +39 0521 905433; fax: +39 0521 905557.
E-mail address: antonella.cavazza@unipr.it (A. Cavazza).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.06.048

[23]. Much attention has been put on the development of the interface between the separation and the detection systems, and the most useful one seems to be the co-axial sheath liquid interface, which employs a sheath liquid to ensure current continuity at the capillary end, also with the aim to improve the ionization process occurring at the interface level [24].

The use of mass spectrometer as detector enhances the potentiality of capillary electrophoresis since it allows to extract the ions matching with the target compound even when complete separation between analytes is not achieved. This is quite convenient when the analysis mixture is rich and complex such as often in biological media. Moreover, mass spectrometry permits the detection of analytes with little or moderate UV absorbance, such as amino acids, without the need of derivatization. Another advantage in using capillary electrophoresis for the analysis of complex matrices lies in the possibility of reducing preliminary pre-treatments of body fluids; in fact, an empty capillary, without stationary phase as in HPLC presents reduced risks of column contamination and in many cases it can be regenerated several times by an easy exhaustive rinse. Moreover, very limited amount of samples are required to be injected in the capillary, and this is very advantageous when low quantities of biological fluids are available. On the other hand, the little amount injected is the reason for a poor concentration sensitivity that represents a major limit of this technique.

Substance P has been only scarcely analysed by CE–MS (e.g., [25]), while some methods for amino acid quantitations have been proposed in the late years employing this technique [26–29]. To our knowledge, this paper presents for the first time the development and validation of a CE–MS method for the simultaneous determination of substance P and of its component free amino acids in biological samples. The human osteosarcoma cell line SaOS-2 was used as enzyme source [30] to model the inactivation of the bone-reactive substance P by bone cell-expressed enzymes. Indeed, preliminary experiments indicated a particularly fast hydrolysis in the presence of these cells, which thus appear particularly suitable for the intended purpose.

2. Experimental

2.1. Materials

Substance P was obtained from Bachem AG (Bubendorf, CH); acetic acid, formic acid, hydrochloric acid, trifluoroacetic acid, ammonium hydroxide, sodium hydroxide, methanol, all amino acids and leucine-enkephaline were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were of HPLC grade.

2.2. Cells and incubation with substrate

Human osteosarcoma line SaOS-2 cells (obtained from IST Genova, Italy) were seeded in flasks of 25 cm^2 and cultured until confluence under standard conditions in Dulbecco medium (Euroclone, London, UK) supplemented with 10% of foetal calf serum (Gibco, Grand Island, NY, USA), 2 mM glutamine and 10 units/mL of penicillin and streptomycin (Sigma Chemical Co., St. Louis, MO, USA). Confluent cells thermostated at 37 °C were washed twice with 140 mM NaCl, 10 mM Na phosphate pH 7.2 (PBS) and then covered with 1 mL of the same buffer. Substance P (3.7×10^{-7} mol) was then added and left for different reaction times from 0 to 600 s (5, 10, 20, 100, 250 and 600 s). All reactions were performed in triplicate, stopped by adding 0.05 mL of 0.05% trifluoroacetic acid, and removing cell supernatants. Samples were stored at −20 °C after evaporation by rotating evaporator.

Before analysis, evaporated reaction mixtures were diluted to 0.25 mL with a solution of formic and acetic acids 50 mM each, at pH 2.7 containing fixed amounts of each internal standard at a final concentration of 5 ppm. Hystidine was selected as internal standard for amino acid quantification, while Leu-enkephaline was chosen as internal standard for substance P evaluation. Solutions were filtered through 4 mm nylon syringe filters 0.25 μm (Millipore).

2.3. Electrophoretic conditions

Samples and standards solutions, prepared by dilution with the background electrolyte, have been analysed by an Agilent Capillary Electrophoresis system (Agilent Technologies, Santa Clara, CA, USA) coupled to mass spectrometer MSD Trap XCT Ultra ion trap with electrospray interface (ESI). Injection time was set at 5 s by applying a pressure of 50 mbar.

Electrophoretic separations were performed employing either a 90 cm or a 60 cm uncoated fused silica capillaries (Agilent Technologies, Santa Clara, California) of 50 μm i.d. and 360 μm o.d., thermostated at 28 °C. CE–MS coupling was realized by a co-axial sheath liquid interface with 50% MeOH and 0.1% formic acid as a sheath liquid at a flow rate of 3 $\mu L\,min^{-1}$.

The running buffer (background electrolyte, BGE) was constituted of an equimolar mixture of formic acid and acetic acid 50 mM, at pH from 2.5 to 5.0 adjusting the desired value with 0.5 M ammonium hydroxide. Applied voltage for separation was 25 kV. Detector was put at the cathodic end.

New capillaries were pre-treated using 0.1 M hydrochloric acid for 5 min, ultrapure water (resistivity = 18 Megohm × cm) for 5 min, 1 M sodium hydroxide for 5 min, ultrapure water for 5 min. Conditioning rinse with the BGE was performed before each session for 20 min. Between runs the capillary was rinsed with BGE for 2 min by applying a pressure of 900 mbar.

When biological samples were injected, an additional rinse was necessary for each five runs to avoid a strong noise in the baseline, often followed by current drop. The rinse consisted in ultrapure water for 5 min, 1 M ammonium hydroxide for 5 min, a mixture of water:methanol 50:50 (v/v) for 5 min, water for 5 min and buffer electrolyte for 15 min.

2.4. Mass spectrometry conditions

Direct infusion experiments, with the goal of optimizing MS parameters for substance P detection, were performed recording the spectra in the range 200–1450 *m/z*. After preliminary experiments, main parameters were selected as follows: electrospray conditions employed nitrogen as nebulizer gas at 10 psi, and drying gas at 10 L/min, 250 °C; positive ionization mode was achieved by the application of 3600 V on the inlet of the mass spectrometer; cone voltage was set at 40 V. Mass spectrometric acquisition parameters for simultaneous substance P, leu-enkephaline and amino acids detection were: full scan mode in the range 60–1450 *m/z*, and subsequent ion extraction corresponding to the specific parent ion of the peptides and each amino acid. Substance P and amino acids quantitation was also achieved by single ion monitoring (SIM) of the molecular ion of each amino acid, and of the signal at 674.5 *m/z*, corresponding to the double charged substance P molecular ion; leu-enkephaline was monitored at 556.4 *m/z*.

Confirmation of peak identities was made by comparison with results obtained by injection of external standards, and also performing the acquisition by transition reaction monitoring mode (MS–MS) choosing the detection of the specific fragments obtained by ulterior fragmentation of each ion.

2.5. Quantitative analysis and method validation

The analytical signal considered was the value of peak area/peak area of internal standard (A/AIS). Linearity was established for

Table 1
Calibration range, regression equation, regression coefficient (R), LOD and LOQ, *m*/*z* ion extracted values after ESI(+) MS-full-scan mode (*m*/*z* range 60–1450) of the considered analytes.

	Range (μmol/L)	Regression equation	R^2	LOD (μmol/L)	LOQ (μmol/L)	*m*/*z*
Substance P	0.3–15	$y = 0.217x - 0.176$	0.9999	0.02	0.06	674.5
Arginine	15–700	$y = 0.273x + 0.749$	0.9991	0.86	2.87	175
Glutamine	15–700	$y = 0.073x - 0.334$	0.9984	2.34	7.80	147
Glycine	35–1000	$y = 0.024x - 0.006$	0.9969	8.30	27.67	76
Leucine	15–700	$y = 0.040x + 0.023$	0.9969	0.64	2.13	132
Lysine	15–700	$y = 0.046x + 0.031$	0.9997	2.15	7.17	147
Methionine	15–700	$y = 0.030x + 0.676$	0.9996	2.14	7.13	150
Phenylalanine	15–700	$y = 0.145x + 0.037$	0.9996	0.94	3.13	166
Proline	25–800	$y = 0.042x + 0.099$	0.9981	5.36	17.87	116

substance P and each amino acid over two orders of magnitude of concentration. The detection limit (LOD) and quantitation limit (LOQ) were calculated as signals based on the mean blank and the standard deviation of the blank signals (3SD for LOD and 10SD for LOQ). Quantitative analyses were performed using calibration curves built by internal standard method. Standard solutions, plus internal standards, were injected at six different concentrations within the range specified in Table 1. Each injection has been made in triplicate and mean values have been evaluated.

Intra-day precision has been evaluated on migration times and peak areas on ten analyses performed in the same day. Inter-day precision has been evaluated on migration times and peak areas on ten analyses performed over three different days. Matrix effect was established by analyzing a blank sample constituted by 1 mL of PBS buffer left inside a cell flask during 600 s, and then processed as described in Section 2.2. The resulting solution was spiked with the analytes standard mixture at two different concentrations, reaching a value corresponding to the second and the fourth level of the calibration curve.

3. Results and discussion

3.1. Separation method optimization and MS acquisition

We planned to find a method able to analyze substance P and its component amino acids under the same analytical conditions. Substance P is an undecapeptide presenting Arg, the most basic amino acid, as N-terminal. In this peptide, three groups are obtainable in their positive charged form at pH < 9.0: the Lys residue, the Arg side chain, and the $-NH_3$ terminal [31]. Such strong basic properties led to the choice of an acidic buffer for capillary electrophoresis analysis, to avoid any interaction of the positive charged analyte with the inner capillary surface; in fact, at low pH value, silanols of the capillary walls are protonated and became neutrals. Furthermore, since pI values of substance P-composing amino acids are in the range between 5.48 and 10.76, the employment of a background electrolyte (BGE) at acidic pH permitted to obtain in their positive charged forms all free amino acids eventually present, thus allowing them migrating towards the cathodic end of the capillary. Moreover, the employment of a low-pH buffer enhances mass spectrometric detection in positive mode [28].

Buffer composition has been optimized by combining formic and acetic acids in order to cover a pH from 2.5 to 5.0 adjusting the desired value with addition of ammonium hydroxide. This equimolar mixture at pH 2.5 has been proposed by Benavente et al. [25] for substance P and more metabolites analysis in urine. We did not change buffer composition and concentration since it was reported from the same authors that resolution obtained employing the two acids together was better than that obtained using one of the acid alone with the same pH and ionic strength. Moreover, buffers at lower concentration leads to loss of efficiency [25,26] while higher concentration lead to longer analysis time [32] and higher current value subsequent to Joule heating, thus limiting the entity of the voltage to be applied [25].

To avoid any possible interaction of the analytes with the inner walls of the capillary, thus leading to fluctuations in the electroosmotic flow (EOF) and irreproducible migration times, different buffers at pH values between 2.5 and 5.0 were investigated. Experiments performed at pH 4.0 and 5.0 did not give good results as broadened peaks for basic amino acids (Arg and Lys) were recorded. No good reproducibility of peak area for substance P was achieved, probably due to interactions with the deprotonated capillary silanol groups [26]. Furthermore, self-association phenomena of substance P probably may occur [33]. For this reason the optimization was focalized in the pH range between 2.5 and 3.0. At pH 2.7 higher stability in terms of peak shape and migration time precision was obtained, demonstrating the absence of uncontrollable alterations on the capillary surface, as well as peptide absorption responsible for little changes in EOF and migration time shifts [34].

The addition of organic modifiers in the running buffer has been suggested by some authors [28,29] for its property of helping the ionization process at the ESI level. We employed methanol only in the composition of the sheath liquid, at a percentage of 50% in water plus 0.1% of formic acid to help the ionization process occurring at the interface level [26,27]. Higher amount of methanol determined drop of current. Water/isopropanol 40:60 (v/v) plus 0.1% of formic acid was also employed but a diminishment of the signal/noise ratio was observed.

UV detection at 210 nm was also employed in the study of the buffer composition optimization. Subsequently, with the aim of reducing analysis time we decided to shorten the capillary avoiding it to pass through the UV window, and using only mass spectrometry for detection. In this way, capillary length was reduced from 90 cm to 60 cm and the analysis time was sensibly reduced. With the new capillary, under the selected conditions, at a voltage of 25 kV and a temperature of 28 °C, substance P migrated at approximately 3 min. Under the same conditions, we performed the analysis of the pentapeptide Leu-enkephaline, chosen as internal standard. Leu-enkephaline has only one basic group, and at pH 2.7 it has a single positive charge on the amino-terminal that makes the molecule migrating to the cathode. Its mobility (6.15E−04 $cm^2\ s^{-1}\ V^{-1}$, migration time about 3.9 min) is lower than that of substance P (8.00E−04 $cm^2\ s^{-1}\ V^{-1}$, migration time about 3 min) since it has a lower mass but only a single charge, whereas substance P has a mass almost double, with three charges distributed on it (lower ratio *m*/*z*). In Fig. 1 the ion traces, extracted at 556.4 *m*/*z* for Leu-enkephaline, and at 674.5 *m*/*z* for substance P, are reported.

Amino acids composing substance P, plus hystidine, chosen as internal standard, appeared to be well detected at the same conditions optimized for substance P analysis, and all of them migrated in a total time of 10 min. In Fig. 2 the extracted ion electropherograms for each amino acid, at a concentration of 10 ppm, are reported

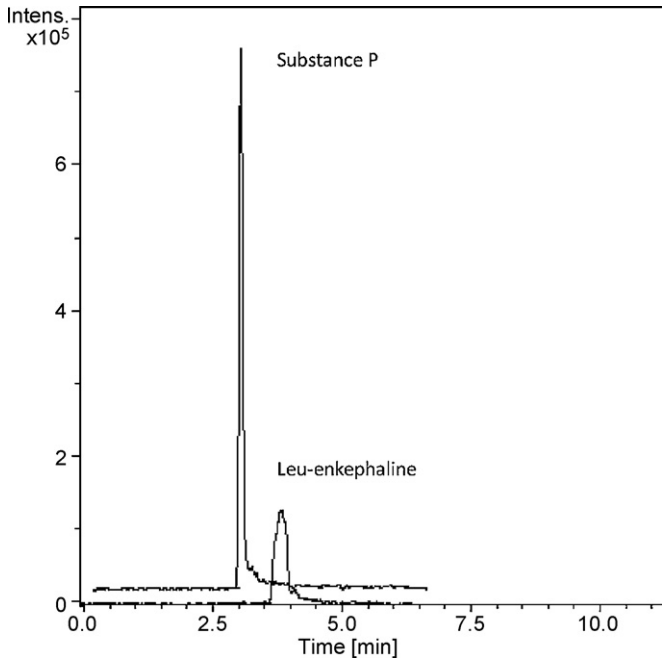

Fig. 1. Extracted ion electropherograms of substance P (674.5 m/z) and Leu-enkephaline (556.4 m/z, internal standard) at a concentration of 5 mg/L. Capillary length 60 cm, applied voltage 25 kV, temperature 28 °C, injection time 5 s. Background electrolyte: formic acid and acetic acid 50 mM each, pH 2.7. Sheath liquid: 50% MeOH, 0.1% formic acid, flow rate 3 μL min^{-1}. ESI(+) full scan mode in the range 60–1450 m/z. Capillary voltage: 3.6 kV; cone voltage 40 V; nebulizer gas 10 psi; drying gas 10 L/min, 250 °C.

(extracted ions for amino acids are shown in Table 2). Migration order corresponded to the theoretical estimation based on ion mobility taking into account the entity of charge and the molecular mass. In Table 2, experimental and calculated electrophorectic data of studied compounds are reported.

After analyzing few biological samples obtained by incubation of substance P with cells, disturbed baseline and drop of current were observed. The reason was probably linked to hydrophobic substances contained in the sample and possibly adsorbed onto the capillary wall which at the low pH employed was neutral. An exhaustive rinse of 5 min employing a solution of water/methanol

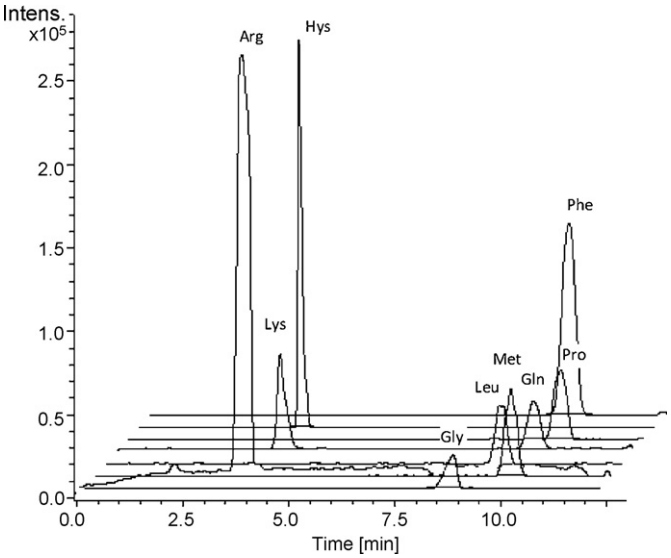

Fig. 2. Extracted ion electropherograms of the analysed amino acids at a concentration of 10 mg/L; experimental conditions as in Fig. 1.

Table 2
Electrophoretic parameters of the considered analytes.

	Migration time (min)	Electrophoretic mobility, μ ($cm^2\ s^{-1}\ V^{-1}$)	Diffusion coefficient[a] ($cm^2\ s^{-1}$)
Substance P	3.0	8.00E−04	5.01E−04
Leu-enkephaline	3.9	6.15E−04	2.05E−03
Arginine	4.0	6.00E−04	1.90E−03
Glutamine	9.8	2.45E−04	6.75E−05
Glycine	8.6	2.79E−04	3.41E−04
Hystidine	4.2	5.71E−04	5.08E−04
Leucine	9.3	2.58E−04	4.00E−04
Lysine	4.1	5.85E−04	1.40E−03
Methionine	9.7	2.47E−04	2.79E−04
Phenylalanine	9.8	2.45E−04	2.70E−04
Proline	10.0	2.40E−04	2.54E−04

[a] Calculated from $D = (\mu \times V/N)/2$.

50:50 (v/v) between runs, followed by a 5 min reconditioning rinse with the running BGE buffer avoided this problem. At the end of each day the capillary was washed with water for 5 min and dried. ESI allows detection of solutes with high molecular mass due to online multiple charge ion formation. The mass spectrometric fragmentation pattern of the protonated substance P ions has been extensively studied by Qin and Yuan [35] and was found to depend on many factors, mostly on the charge status and the fragmentation conditions. In order to optimize MS parameters we performed a tuning during direct infusion of a solution 0.1 mg/mL, scanning between 200 and 1450 m/z. The observed spectrum, reported in Fig. 3, contained in higher abundance the signal of the double charged ion at $m/z = 674.5$. The position of the first charge of the peptide is definitely located on the Arg segment, which is the most basic amino acid, while the second charge could be attributed to Lys segment, although it has been hypothesized that a mobile charge between Lys and Pro residues can occur [35]. Two more double charged species were observed, with $m/z = 600.4$ and $m/z = 543.9$, and can be attributed respectively to the b10 and the b9 ions derived by in-source fragmentation of the entire peptide. One more signal, at $m/z = 1347.8$, is relative to the intact mono charged substance P. The small signal recorded at $m/z = 1086.7$ can be attributed to the singly charged b9 fragment. For quantitative purposes we selected the signal at $m/z = 674.5$ which is the most abundant one.

Leu-enkephaline, selected as internal standard, is a small peptide, composed of five residues. It gets ionized in the ESI assuming a single charge and giving a signal of the protonated molecule ion at $m/z = 556.4$ whose acquisition has been chosen for our quantitative goals.

Mass spectra of all amino acids of interest in positive ionization mode gave always the protonated molecular ion as the predominant signal.

Solutions of standard amino acids composing substance P, plus hystidine selected as internal standard, were injected onto the

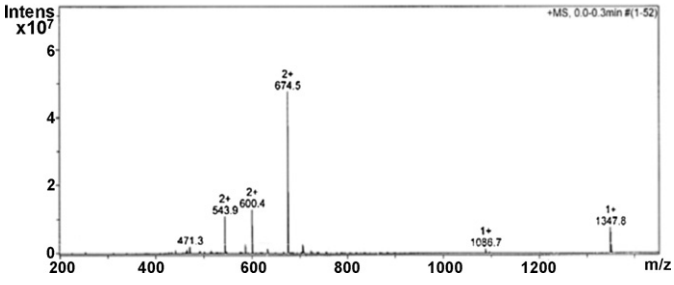

Fig. 3. ESI(+) mass spectrum of an aqueous solution of substance P (0.1 mg/mL); capillary voltage: 3.6 kV; cone voltage 40 V; nebulizer gas 10 psi; drying gas 10 L/min, 250 °C.

capillary and signals were acquired both in full-scan mode (in the range 60–1450 m/z) and in SIM for each m/z value of the amino acid of interest. With the same values of optimized tuning for substance P (reported in Section 2.4), good signals were obtained for all analytes. Glycine showed the lowest sensitivity because of its low molecular weight. High response recorded for arginine could be explained because of its basic characteristics that led to a better ionization in positive ion mode [26]. No significant differences in terms of quantitative results (obtained by calibration curves built from full-scan followed by ion extraction, and by SIM) were observed. Therefore, to acquire all signals in a single run, we performed the acquisition in full-scan mode.

3.2. Validation and quantitative analysis

In order to perform quantitative analyses we checked the linearity of signal responses obtained in the range of concentration of interest, as reported in Table 1, together with LOD and LOQ values expressed for each analyte. Calibration curves were built as described in Section 2, and correlation coefficients resulted always higher than 0.997. Intra-day and inter-day repeatability of migration times were calculated on ten analyses performed in the same day or over three different days. Values for relative standard deviation (RSDs%) obtained for migration times were between 0.66 and 2.39% for intra-day measures, and between 0.75 and 3.15% for inter-day measures. About absolute peak areas, RSD% values were between 1.91 and 6.50% for intra-day, and between 1.85 and 7.30% for inter-day. When peak areas were corrected by internal standard all values remained always below 4.80%. Obtained results for matrix effect, established as described in Section 2.5, and considering the A/AIS values, were within RSD% = ±9 for all compounds.

Measures on blank samples were performed in order to evaluate the baseline noise. Small signals at m/z corresponding to phenylalanine were detected, and their identity could be confirmed by MS–MS analysis, by monitoring the transition 116 → 70.3. This datum suggests that small amount of this amino acid was present in the cell media, and quantitative data recorded for phenylalanine could be affected by a negligible overestimation, since the amount found in the blank sample was below the calculated LOQ for this amino acid.

Quantitative analyses were performed on samples obtained after incubation of substance P with SaOS-2 cells for periods of times ranging from 0 to 600 s and monitoring the amount of intact substance P and of free amino acids. The steady decrease of substance P as function of reaction time indicated by the data shown in Fig. 4 is consistent with substrate hydrolysis catalyzed by cell-expressed enzymes. On the basis of these data, substance P (extrapolated) half-life was 4.3 s, indicating an extremely fast hydrolysis in the presence of the SaOS-2 cells. The decrease of the intact substrate was accompanied by a noticeably slower appearance of free, substance P component amino acids (insert in Fig. 4). Together with the above-referred ineffectiveness of exopeptidases in catalyzing substance P hydrolysis, this datum can be interpreted as indicating that free amino acids derive from secondary, exopeptidase and endopeptidase catalyzed, hydrolysis of peptidic fragments released by endopeptidases (data submitted). The amount found for each amino acid is function of its position relative to the attack points, of its release kinetics, and of its relative amount in the substrate. The data shown are consistent with the hydrolysis of the Pro4-Gln5 bond, followed by sequential degradation of the Arg-Pro-Lys-Pro fragment; with the hydrolysis of the Phe7-Phe8 bond, leading to release of Phe and Gln; with the hydrolysis of the Gly9-Leu10 bond, with release of free Met and Leu from the C-terminal dipeptide.

On a functional standpoint, although the actual concentrations of substance P in bone are presently unknown, the existence – at least in some tissue – of a hydrolysis as fast as that reported here may contribute to explain the high concentrations of this peptide described as necessary to induce biological effects [36]. However, it seems also necessary to observe that the biological activity of substance P's terminal fragments [17,37] may affect the results of this kind of analyses. Finally, because of the very limited amount of the relevant enzymes, substance P concentrations possibly higher in our experimental set-up than the actual (unknown) in vivo concentrations could only underestimate its hydrolysis with respect to the in vivo conditions.

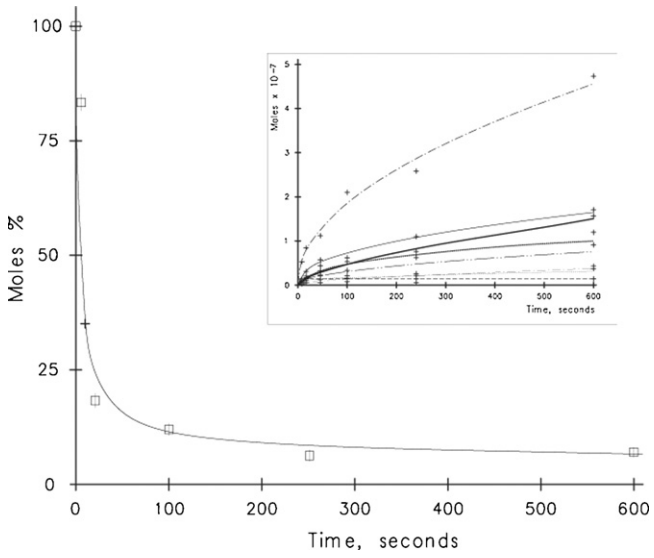

Fig. 4. Disappearance of substance P in the presence of SaOS-2 cells at reaction times between 0 and 600 s, moles percent; thin vertical lines represent error bars ($n = 3$; t-Student significance = 95%). Insert: appearance in the reaction medium of free amino acids released from the fragments deriving from primary substrate hydrolysis; data expressed as absolute moles.

4. Conclusions

The developed method permitted the simultaneous evaluation of substance P and its component free amino acids under the same analytical conditions, without modifying mass spectrometric acquisition parameters. The migration of all species of interest was performed in about 10 min. The proposed approach was applied to the analysis of biological samples obtained from incubation of substance P with an osteosarcoma cell line, and permitted to follow the enzymatic kinetics of substance P disappearance during 600 s to follow its hydrolysis in the presence of living cells.

References

[1] U.S. von Euler, J.H. Gaddum, J. Physiol. 72 (1931) 74.
[2] C. Severini, G. Improta, G. Falconieri-Erspamer, S. Salvadori, V. Erspamer, Pharmacol. Rev. 54 (2002) 265.
[3] T. Goto, T. Tanaka, Microsc. Res. Technol. 58 (2002) 91.
[4] L. Liu, E. Burcher, Peptides 26 (2005) 1369.
[5] T. Cao, E. Pinter, S. Al-Rashed, N. Gerard, J.R. Hoult, S.D. Brain, J. Immunol. 164 (2000) 5424.
[6] D. Liu, L.-S. Jiang, L.-Y. Dai, Neuropeptides 41 (2007) 271.
[7] J.E. Keeble, S.D. Brain, Neurosci. Lett. 361 (2004) 176.
[8] L. Wang, S. Ahmad, I.F. Benter, A. Chow, S. Mizutani, P.E. Ward, Peptides 12 (1991) 1357.
[9] M.A. Adamus, Z.J. Dabrowski, J. Cell. Biochem. 81 (2001) 499.
[10] G.N. Onuoha, Peptides 22 (2001) 1107.
[11] N.M. Hooper, J. Hryszko, A.J. Kenny, Biochem. J. 267 (1990) 509.
[12] P.E. Ward, in: R.M. Burch (Ed.), Bradykinin Antagonists: Basic and Clinical Research, Marcel Dekker, New York, 1991, p. 147.
[13] R. Matsas, I.S. Fulcher, A.J. Kenny, A.J. Turner, Proc. Natl. Acad. Sci. U.S.A. 80 (1983) 3111.
[14] R.A. Skidgel, S. Engelbrecht, A.R. Johnson, E. Erdos, Peptides 5 (1984) 769.

[15] S.L. Stephenson, A.J. Kenny, Biochem. J. 241 (1987) 237.
[16] S. Blumberg, V.I. Teichberg, J.L. Charli, L.B. Hersh, J.F. McKelvy, Brain Res. 192 (1980) 477.
[17] R.W. Bury, M.L. Mashford, J. Med. Chem. 19 (1976) 854.
[18] J.M. Stewart, M.E. Hall, J. Harkins, R.C.A. Frederickson, L. Terenius, T. Hokfelt, W.A. Krivoy, Peptides 3 (1982) 851.
[19] C. Simò, C. Barbas, A. Cifuentes, Electrophoresis 26 (2005) 1306.
[20] G. Font, M.J. Ruiz, M. Fernandez, Y. Picò, Electrophoresis 29 (2008) 2059.
[21] P. Zurbig, E. Schiffer, H. Mischak, Methods Mol. Biol. 564 (2009) 105.
[22] A. Vassort, D.A. Barrett, P.N. Shaw, P.D. Ferguson, R. Szucs, Electrophoresis 26 (2005) 1712.
[23] C. Desiderio, D.V. Rossetti, F. Iavarone, I. Messana, M. Castagnola, J. Pharm. Biomed. Anal. 53 (2010) 1161.
[24] M.R.N. Monton, S. Terabe, Anal. Sci. 21 (2005) 5.
[25] F. Benavente, R. van der Heijden, U.R. Tjaden, J. van der Greef, T. Hankemeier, Electrophoresis 27 (2006) 4570.
[26] T. Soga, D.N. Heiger, Anal. Chem. 72 (2000) 1236.
[27] N. Shama, S.W. Bai, B.C. Chung, B.H. Jung, J. Chromatogr. B 865 (2008) 18.
[28] A.V. Colnaghi Simionato, E.P. Moraes, E. Carrilho, M.F. Maggi Tavares, E. Kenndler, Electrophoresis 29 (2008) 2051.
[29] O.A. Mayboroda, C. Neususs, M. Pelzing, G. Zurek, R. Derks, I. Meulenbelt, M. Kloppenburg, E.P. Slagboom, A.M. Deelder, J. Chromatogr. A 1159 (2007) 149.
[30] S.B. Rodan, Y. Imai, M.A. Thiede, G. Wesolowski, D. Thompson, Z. Bar-Shavit, S. Shull, K. Mann, G.A. Rodan, Cancer Res. 47 (1987) 4961.
[31] C-S.C. Wu, A. Hachimori, J.T. Yang, Biochemistry 21 (1982) 4556.
[32] W. Lu, G. Yang, R.B. Cole, Electrophoresis 16 (1995) 487.
[33] M. Rueger, M. Bienert, B. Mehlis, K. Gast, D. Zirwer, J. Behlke, Biopolymers 23 (1984) 747.
[34] P.S. Kopplin, A.V. Garmash, A.V. Kudryavtsev, F. Menzinger, I.V. Perminova, N. Hertkorn, D. Freitag, V.S. Petrosyan, A. Kettrup, Electrophoresis 22 (2001) 77.
[35] X.-Z. Qin, Y. Yuan, Int. J. Mass Spectrom. 237 (2004) 123.
[36] T. Mori, T. Ogata, H. Okumura, T. Shibata, Y. Nakamura, K. Kataoka, Biochem. Biophys. Res. Commun. 262 (1999) 418.
[37] M.E. Hall, F. Miley, J.M. Stewart, Peptides 10 (1989) 895.