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ORIGINAL ARTICLE

Quantification of thyrotropin-releasing hormone by liquid chromatography–electrospray mass spectrometry

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Abstract Thyrotropin-releasing hormone (TRH) is involved in a wide range of biological responses. It has a central role in the endocrine system and regulates several neurobiological activities. In the present study, a rapid, sensitive and selective liquid chromatography–mass spectrometry method for the identification and quantification of TRH has been developed. The methodology takes advantage of the specificity of the selected-ion monitoring acquisition mode with a limit of detection of 1 fmol. Furthermore, the MS/MS fragmentation pattern of TRH has been investigated to develop a selected reaction monitoring (SRM) method that allows the detection of a specific b2 product ion at *m/z* 249.1, corresponding to the *N*-terminus dipeptide pyroglutamic acid–histidine. The method has been tested on rat hypothalami to evaluate its suitability for the detection within very complex biological samples.

Keywords Thyrotropin-releasing hormone · Liquid chromatography · Mass spectrometry · Selected-ion monitoring · Selected reaction monitoring

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List of abbreviations

DMF	Dimethylformamide
DCM	Dichloromethane
HBTU	1-H-Benzotriazolium, 1-[bis(dimethylamino)methylene]-hexafluorophosphate(1-), 3-oxide
HOBt	*N*-hydroxybenzotriazole
DIEA	Di-isopropylethylamine
Fmoc	Fluorenylmethoxycarbonyl
<Glu	Pyroglutamic acid
TFA	Trifluoroacetic acid
TIS	Tri-isopropylsilane
TRH	Thyrotropin-releasing hormone

Introduction

In the previous years several studies have been focused on the detection of bioactive peptides content within organisms and tissues (peptidomics). In particular, great efforts have been made to identify, characterise and quantify neurotransmitters, neuropeptides and various hormones (Strand 2003; Svensson et al. 2003; Che et al. 2005a; Fricker et al. 2006).

Hypothalamus has been frequently chosen as a model system in peptidomic analyses for its high content of neurohormones and neuropeptides and for its crucial role in regulating the endocrine system (Che et al. 2005, b; Decaillot et al. 2006). However, the analysis of endogenous neuropeptides is a very challenging task due to their great diversity and their low concentrations within complex matrices.

Thyrotropin-releasing hormone (TRH, <Glu–His–Pro–NH_2) was the first hypophysiotropic peptide to be isolated from the hypothalamus (Boler et al. 1969; Burgus et al. 1970). It plays a pivotal role in stimulating thyrotropin

(TSH) release from the mammalian anterior pituitary gland. Furthermore, the broad phylogenetic and phenotypic distribution of TRH suggests that it may possess additional, although not yet fully elucidated, functions other than regulation of the release of pituitary hormones. Several clinical studies support the evidence that TRH acts as a neuroregulator and/or neuromodulator in the central nervous system of mammals (Kastin et al. 1972; Prange et al. 1972; Lechan and Fekete 2006; Monga et al. 2008; Shibusawa et al. 2008). These findings are also supported by the occurrence of many TRH analogues endowed with antidepressant, euphoric, and neuroprotective properties (Bilek 2000; Hinkle et al. 2002; Sattin et al. 2003). In addition, it has been demonstrated that higher amounts of stimulatory neuropeptides, including TRH, are present in pituitary adenomas compared with the normal pituitary glands (Peillon et al. 1989; Pagesy et al. 1992). Additional functions of TRH are related to the reproductive system, where, by regulating the development of Leydig cells before maturation and the secretion of testosterone after maturation, it plays a role in testicular functions (Zhao et al. 2008).

Thyrotropin-releasing hormone identification and quantification have been classically based on radioimmunoassays (RIA) (Visser et al. 1974; Visser et al. 1977; Nillni et al. 2000), but this technique, although powerful in terms of sensitivity, suffers a very poor specificity due to the presence of closely related peptides. TRH determination, therefore, often requires additional techniques to further characterise the immunogenic material. Analytical approaches that couple peptide separation (e.g. reversed-phase high-performance liquid chromatography, multidimensional chromatographic techniques and capillary electrophoresis) and detection (absorbance, fluorescence and electrochemistry) have been widely used in the past for TRH and TRH-like peptides analysis (Spindel and Wurtman 1979, 1980; Busby et al. 1981a, b; Spindel et al. 1981; Sandberg and Weber 2003; Warner and Weber 1989). Nevertheless, UV and electrochemical detections are both characterised by an overall low sensitivity and fluorescence requires derivatisation on functional groups (e.g. primary amines or thiols, which are typically lacking in small peptides such as TRH (Spindel et al. 1981). An online preconcentration method to enrich TRH through adsorption on SDS-modified precolumns has also been proposed (Meng et al. 2005). The technique employed both microbore and capillary HPLC coupled with the electrochemical detection.

Mass spectrometry (MS) coupled to liquid chromatography (LC–MS) has proven to be a powerful tool in qualitative and quantitative analyses of peptides and neuropeptides, including two opioid neuropeptides (methionine enkephalin and β-endorphin) (Dass and Desiderio 1987; Desiderio 1996a; b; Desiderio and Zhu 1998; Holm et al. 2005; Tamvakopoulos 2007). Althought the use of MS applied to TRH analysis dates back to 1970, when its primary structure was determined by electron ionisation (EI) and chemical ionisation (CI) techniques, very little work on the quantitative determination of TRH by MS has been reported (Burgus et al. 1970; Beuhler et al. 1972; Desiderio 1996b). Therefore, the development of a simple detection method with high sensitivity and selectivity for TRH identification and quantification is still a need for understanding the physiological processes involving TRH- and TRH-like peptides.

In the present study, we report a rapid, sensitive and selective LC–MS method for the identification and quantification of TRH. The described methodology utilises positive electrospray ionisation (ESI) on a single quadrupole mass spectrometer in "selected-ion monitoring" (SIM) mode. To further improve the specificity of the analysis, a transition in selected reaction monitoring (SRM) mode has also been investigated.

To the best of our knowledge, this is the first report in which MS in SIM and SRM acquisition modes have been applied to the qualitative and quantitative analysis of TRH, whose determination is of utmost importance in neuroscience, biochemical and clinical studies.

Materials and methods

Chemicals and materials

Acetonitrile was purchased from Carlo Erba (Milan, Italy). C_{18} Ultrasphere ODS columns (4.6 × 150 mm, 5 µm) were purchased from Beckman Coulter (CA, USA). All chemicals for peptide synthesis, including resins, were from Novabiochem (Laufelfingen, CH). Formic acid, trifluoroacetic acid, dimethylformammide (DMF), dichloromethane, diethylether and methanol were all from LabScan (Dublin, Ireland). Chemicals and solvents for the automated amino acid analysis were obtained from Biochrom (Cambridge, UK). Nor-Leu was purchased from Sigma Chemical Co. (Milan, Italy).

Peptide synthesis

Solid-phase chemical synthesis of TRH was accomplished on a 5 µmol scale following the Fmoc strategy and using standard Fmoc-derivatised amino acids (Fields and Noble 1990). RINK AMIDE resin (substitution 0.5 mmol/g) was used as a solid support. Activation of amino acids was achieved using HBTU/HOBt/DIEA (1:1:2), whereas Fmoc deprotection was carried out using a 20% (v/v) piperidine solution in DMF. All couplings were performed for 15 min

and deprotections for 10 min. The peptide was removed from the resin by treatment with a TFA:TIS:H_2O (90:5:5, v/v/v) mixture, then it was precipitated in cold diethylether and lyophilised. TRH purification was performed by following a conventional RP-HPLC approach. The purified TRH was dried under vacuum and then dissolved in deionised H_2O and aliquoted. Standard solutions were stored at −20°C until use.

Amino acid analyses

Amino acid analyses for the quantification of TRH to be used as a standard were performed as previously described (Del Vecchio Blanco et al. 1997). Briefly, aliquots (25 µL) of synthetic THR were hydrolysed in triplicate at 110°C for 20 h with 300 µL of 6 N hydrochloric acid containing 0.02% phenol and nor-Leu (20 nmol) as internal standard. Following hydrolysis, HCl was removed under vacuum and samples resuspended in 0.5 mL of 0.2 M lithium citrate buffer (pH 2.2). Aliquots (100 µL) were then analysed using a Biochrom 20 amino acid analyser (Biochrom, Cambridge, UK) equipped with a polyvinyl sulphonate ionic exchange column (Biochrom, Cambridge, UK) and a post-column ninhydrin derivatisation system. The detection was performed both at 570 and 440 nm for proline detection.

Liquid chromatography conditions

Optimisation of the chromatographic conditions for TRH detection and quantification by RP-HPLC was performed on a Waters Breeze instrument connected to the ESI interface of the mass spectrometer. The LC system was equipped with a Beckman C_{18} Ultrasphere ODS column (4.6 × 150 mm, 5 µm particle size) and an ODS guard column (Security Guard, Phenomenex Inc., Torrance, CA, USA). The chromatographic separation was carried out using water (solvent A) and CH_3CN (solvent B), both containing 0.1% formic acid at a flow rate of 0.5 mL/min and a sample injection volume of 50 µL. TRH retention was achieved by equilibrating the reversed-phase column at 0% solvent B. Peptide elution was then carried out using a short two steps linear gradient of solvent B from 0 to 5% over 1 min and from 5 to 95% of solvent B over 5 min. An isocratic step at 95% of solvent B for 4 min was added to wash the column. Under these conditions, TRH was eluted at 6.5 min with a 15 min equilibration time. All analyses were performed at 25°C.

Mass spectrometric conditions

Mass spectrometry analyses were performed on a Finnigan AQA single-quadrupole MS (ThermoFisher, Milan, Italy) equipped with an ESI source operating in positive-ion mode. Mass spectrometric conditions were optimised to obtain the maximum sensitivity for the TRH precursor ion. The final MS parameters were as follows: the ESI probe temperature, the cone voltage and the capillary voltage were set at 300°C, 20 V and 3.5 kV, respectively. Preliminary acquisitions were made in full-scan in the range *m/z* 200–500. For quantitative determinations, acquisitions were made in SIM mode for TRH (theoretical $[M + H]^+$ monoisotopic value: 363.2), with a dwell time of 0.37 s and a span of 0.1 µm. Data analyses and processing were performed using the Finnigan Xcalibur software (v. 1.3, ThermoFisher, Milan, Italy). Considering the instrumental accuracies, all quadrupole and ion-trap mass values were reported with one decimal place.

Quantitative analysis

Thyrotropin-releasing hormone primary standard stock solution at a concentration of 4.1 mM in deionised water, as determined by amino acid analysis, was used to prepare a set of diluted samples to span a 6-point calibration range between 1 and 1,000 fmoles. The area of the main peak at 6.5 min was used for linear curve construction in SIM mode. Each sample was analysed in duplicate and two blank runs were introduced after each injection to prevent carryover between separate runs. Chromatograms were processed for peak integration using the ICIS peak detection and integration algorithm using default parameters. In particular, a 15 points Gaussian smoothing and a 40 points baseline window were used prior to data processing. In addition, a tolerance of ±0.5 min was set for peak identification. Linear regression analysis was applied to standard calibration curve construction using the QuanBrowser package of Xcalibur software (ThermoFisher, Milan, Italy). Processed data were exported to the GraphPad Prism software (v. 4, GraphPad Software Inc., La Jolla, USA). The integration parameters were set to produce reproducible integration over several runs.

MS/MS experiment on quadrupole/orthogonal-time-of-flight (Q-TOF) mass spectrometer

The fragmentation pattern of TRH was investigated using a Q-TOF Micro mass spectrometer (Waters, Milford, MA USA) fitted with a Z-spray electrospray-ion source (Chambery et al. 2008). Standard TRH at a concentration of 500 fmol/µL was infused into the system at a flow rate of 5 µL/min. The capillary source voltage and the cone voltage were set at 3,000 and 35 V, respectively. The optimal collision energy (CE) value for peptide MS/MS was optimised by varying the CE during infusion and was finally set at 30 V. The source temperature was kept at 80°C and

nitrogen was used as a drying gas (flow rate 50 L/h) and argon as collision gas. The MS/MS data were processed using MaxEnt3 algorithm in MassLynx 4.1 software (Waters S.p.A., Manchester, UK) for de-isotoping and deconvolution. De novo sequencing was obtained by means of the Biolynx application of MassLynx software.

Extraction procedure of TRH from rat hypothalamus

Preweighed rat hypothalami (about 40 mg), as collected, were homogenised with 0.2 M TCA at a 1:10 (w/v) ratio. Under these conditions, proteins (including proteases) are precipitated and/or inactivated. The homogenate was centrifuged at 10,000*g* for 5 min. The supernatant was treated with diethylether (1:20, v:v) to extract TCA. Samples were dried under vacuum and dissolved in 600 µL of deionised water. Aliquots (6 µL) of the hypothalamic extracts were analysed in duplicate. For TRH quantification, the average calculated amounts were multiplied for the dilution factor (1:100) and for theoretical TRH molecular weight (362.2 Da). The recovery of the extraction procedure was also evaluated by determining the TRH content in a hypothalamic extract spiked with 500 pmol of standard TRH before extraction. The spiked sample was processed as described above. Similar conditions were used for processing of rat hypothalami used for quantitative determinations by SRM.

Selected reaction monitoring (SRM) analysis

For acquisitions in SRM mode, LC–MS analyses were performed using an LCQ DECA XP Ion Trap spectrometer (ThermoFisher), equipped with an Opton electrospray ionisation source (operating at a needle voltage of 3.5 kV and at a temperature of 300°C). A Surveyor HPLC system connected to the mass spectrometer was used for chromatographic separation as described above. The SRM acquisition was performed by monitoring the transition of the $[M + H]^+$ precursor ion at *m/z* 363.2 to the specific b2 product ion at *m/z* 249.1, corresponding to the *N*-terminus dipeptide (pyroglutamic acid–histidine). Fragmentation was induced on the selected ion with a fixed 28% of total energy. The integration of chromatographic peaks and the construction of calibration curves were performed as described above.

Results

Standard TRH synthesis and quantification

Solid-phase chemical synthesis of TRH performed using the Fmoc strategy as described in the "Methods" section, afforded the desired tripeptide in quantitative yield. Following the RP-HPLC purification, a stock solution of synthetic TRH was accurately quantified by amino acid analysis (Table 1), resulting at a final concentration of 4.1 mM.

Chromatographic method and optimisation of mass spectrometric conditions

Previous attempts to analyse TRH by conventional RP-HPLC approaches often failed due to its low-molecular weight and high polarity. Indeed, in weak acidic solution TRH is a hydrophilic cation and is not retained on RP-HPLC columns using typical TFA/acetonitrile mobile phase systems (Meng et al. 2005). With the aim to overcome this shortcoming, an LC–MS method for TRH determination was developed. Retention and subsequent elution of TRH from a C_{18} Ultrasphere ODS column were achieved by performing the column equilibration in aqueous mobile phase without the organic solvent. Under these conditions, the tripeptide is retained by the reversed-phase

Table 1 TRH standard quantification by amino acid analysis

Amino acid	Analysis #1 (nmols)	Analysis #2 (nmols)	Analysis #3 (nmols)	Average (nmols)
<Glu	23.43	24.05	22.42	23.30 ± 0.82
His	18.43	19.08	17.76	18.42 ± 0.66
Pro	19.88	20.55	19.07	19.83 ± 0.74
Nor–Leu yield	91.20%	89.70%	94.45%	
			TRH nmols	20.51 ± 2.51 × 5[a] = 102.55/25 µL

Experimental values obtained by acid hydrolysis of synthetic TRH aliquots (25 µL)

A correction has been applied, taking into account the internal standard yield (theoretical amount: 20 nmol)

Corrected values have been used for the average nmols calculation

TRH stock solution concentration was found to be 4.1 nmol/µL (4.1 mM)

[a] A dilution factor of 1:5 was considered since 100 µL out of 500 µL of the resuspended hydrolyzed mixture were used for each analysis

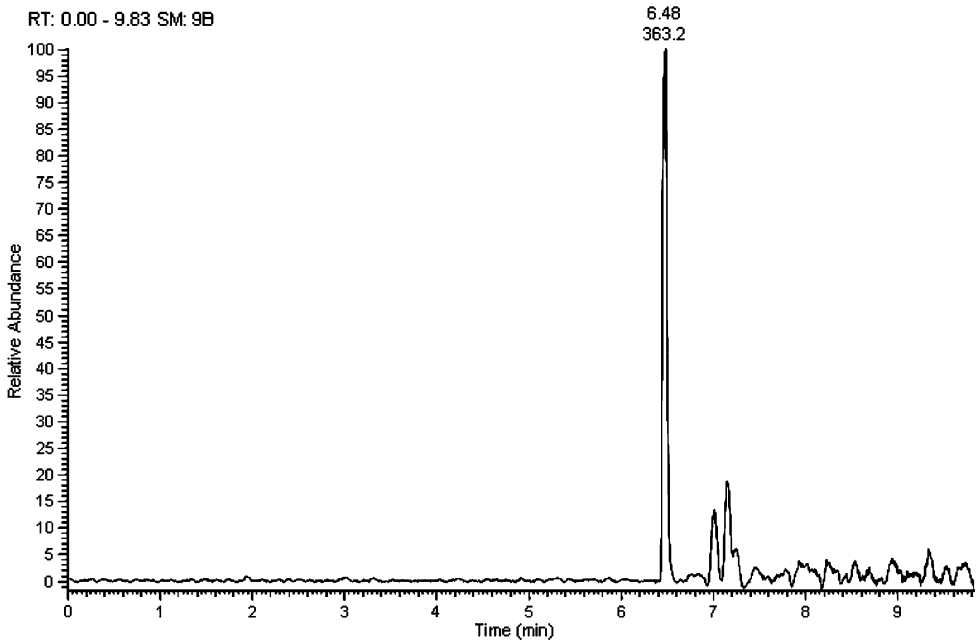

Fig. 1 Representative mass chromatogram acquired in SIM mode of standard TRH (5 pmol). A Gaussian smoothing and baseline subtraction processing were applied on raw data

column. The following application of a very rapid gradient results in the elution of TRH as a sharp peak at an elution time of 6.5 min. In order to improve the sensitivity, the ESI interface parameters of the single quadrupole spectrometer were optimised for TRH molecular ion detection. Therefore, the acquisition parameters were determined by direct infusion into the mass spectrometer of a 500 fmol/μL standard solution in water: acetonitrile (50:50, v: v) containing 0.1% formic acid, at a flow rate of 50 μL/min. The optimisation of ESI parameters was performed on the experimental TRH protonated precursor ion at $m/z = 363.2$ ($[M + H]^+$) that was selected for the following quantitative analyses in SIM acquisition mode. A representative SIM chromatogram referred to standard TRH (5 pmol) is shown in Fig. 1.

TRH quantification in SIM mode

The SIM acquisition mode was used to investigate the linear range and the lower limit of detection of the instrument. Different amounts of standard TRH in the range of 1–1,000 fmol were injected and the area of the peak at 6.5 min was used for reference curve construction (Fig. 2a). The calibration curve reported in Fig. 2b shows that a linear response was obtained over two orders of magnitude with a correlation coefficient of 0.999. Calibration curves were also highly reproducible when replicate injections were performed over several days (data not shown). The lower limit of detection was assumed to be 1 fmol (S/N ratio = 15). Lower amounts were not tested because literature values reported for TRH physiological concentrations are far higher than the assayed concentrations (i.e. about 350 nM in rat hypothalamus) (Winokur and Utiger 1974).

Fragmentation pattern of TRH

The fragmentation pattern of TRH was investigated using a Q-TOF *Micro* mass spectrometer, with the aim to further increase the molecular specificity of the developed methodology. Indeed, in the SIM mode, the presence of the $[M + H]^+$ value corresponding to that of the analyte, indicates only that a peptide with the appropriate molecular weight is eluted at the expected retention time. An improved molecular specificity is provided by "Selected Reaction Monitoring" (SRM) experiments. In the SRM mode, the ion current due to a specific fragment ion deriving from the transition of the $[M + H]^+$ ion is monitored. For this purpose, we investigated the TRH fragmentation behaviour, taking into account a previously reported methodology developed by our group for GnRH detection (Chambery et al. 2008). The method was based on the selective detection of a specific b2 product ion at m/z 249.1, corresponding to the preferential fragmentation of the *N*-terminus dipeptide pyroglutamic acid–histidine (<Glu–His), highly conserved in GnRH as well as several other neuropeptides. Figure 3 reported the tandem mass spectrum of the experimental precursor ion at m/z 363.18, corresponding to the $[M + H]^+$ mass of TRH (expected molecular mass: 362.17 Da; $\Delta = 0.01$. The fragmentation of the tripeptide, driven by the increase in the collision energy to 30 V, clearly revealed the presence of the mass

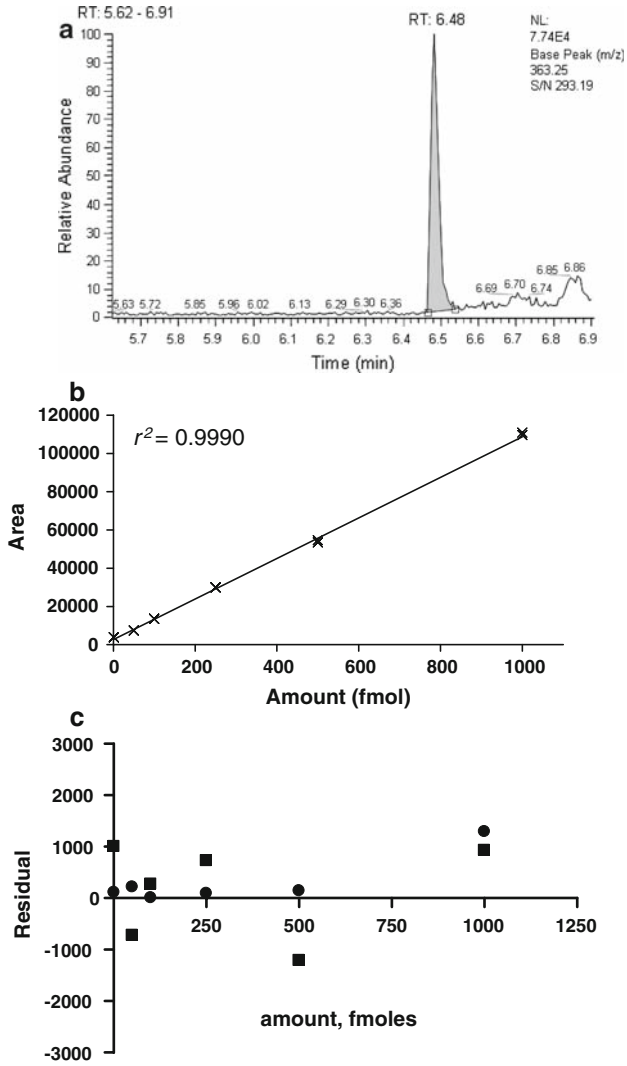

Fig. 2 The extracted ion at *m/z* 363.2 of TRH (**a**) was used for the construction of the calibration curve in SIM acquisition mode (**b**). A linear polynomial type curve was used for data fitting. A linear response was obtained over two orders of magnitude with a correlation coefficient of 0.999. The plot of residuals shows that deviations of experimental data points from the theoretical curve are all below 1% (**c**). *Circles* and *squares* refers to duplicate experimental points

peak at *m/z* 249.1, which is diagnostic of the indicated b2 fragment ion. The formation of the TRH *N*-terminal dipeptide was confirmed under a broad range of collision energies and also by inducing a cone voltage fragmentation (CID) on the ESI source of a single quadrupole mass spectrometer (Fig. 4). By applying a cone voltage ramp in the range 30–35 V, we observed that mainly the diagnostic ion at m/z 249.1 was present in the fragmentation spectrum, along with the parent ion of TRH. These results strongly suggested that the preferential fragmentation producing the <Glu–His dipeptide was very stable and essentially independent from the method and parameters used to induce the fragmentation.

TRH quantification from rat hypothalami

To validate the methodology and also to verify its suitability for the analysis of biological samples, we quantified the physiological TRH in peptide extracts of rat hypothalamus. The SIM mass chromatogram of a representative sample of rat hypothalamus is reported in Fig. 5. A peak at the elution time of TRH was clearly detected in rat extracts. Aliquots of hypothalamic extracts were then analysed in duplicate for the evaluation of physiological levels of TRH. The calculated amount of TRH was found to be 0.22 ± 0.02 pmol/mg of hypothalamus (average value calculated considering the in 4 independent experiments on 2 rats, see Table 2 for details). This result is in very good agreement with the value of 0.3 pmol/mg tissue, previously reported for TRH content in rat hypothalamus (Winokur and Utiger 1974). Control samples spiked with known amounts of standard TRH before extraction (500 pmol) were also analysed to check the reproducibility of the TRH elution time within the extract and to evaluate the recovery of the extraction procedure. The determined recovery was found to be $90\% \pm 5$ (mean $\pm$ SD, $n = 3$).

Selected reaction monitoring (SRM) analysis

As expected, monitoring of TRH in SIM mode was found to significantly increase the signal-to-noise ratio of the analyte peak in the ion chromatograms compared with that acquired in full-scan mode (data not shown). The enhanced sensitivity and specificity of SIM acquisitions derive from the longer dwelling time of the mass spectrometer scan time over a smaller mass range. Nevertheless, as observed in Fig. 5, the SIM plots still presented more than one peak, resulting in the lack of a unique identifier for the compound of interest. These results are typically observed for SIM chromatograms of complex samples, as the likelihood to detect compounds with the same and/or similar molecular masses can be fairly high. Elution time is, therefore, assumed as diagnostic of peak identity.

The highly specific fragmentation pattern of TRH prompted us to set up an SRM experiment to further improve the molecular specificity for the detection and quantification of the hormone. In this instance, the LC–MS analyses were performed also on an LCQ DECA XP Ion Trap spectrometer, equipped with an ESI source. The SRM acquisition was performed as reported in the "Methods" section. A representative mass chromatogram of standard TRH (200 fmoles injected) acquired in SRM mode is reported in Fig. 6A. Considering the physiological concentration of TRH obtained in the previous analysis, in SRM mode, the

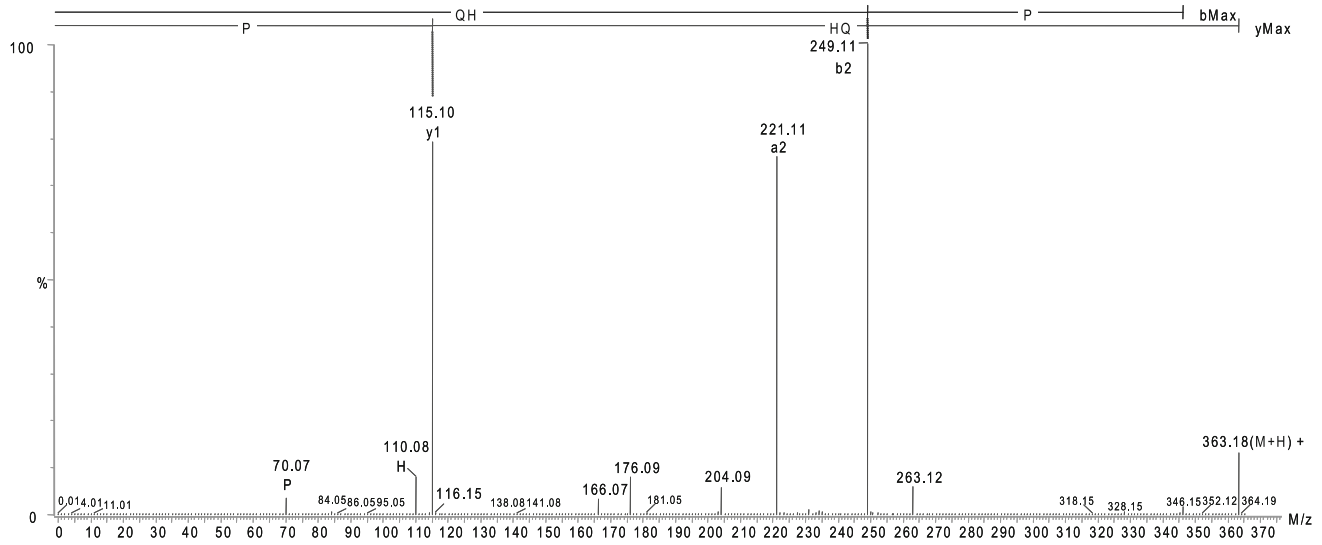

Fig. 3 Product-ion spectrum deriving from the fragmentation of the $[M + H]^+$ TRH precursor ion at *m/z* 363.18. The MS/MS spectrum is annotated with the *y* and *b* ion series from the TRH sequence (<Glu–His–Pro–NH_2). The proline and histidine immonium ions are also present at *m/z* 70.07 and 110.08, respectively

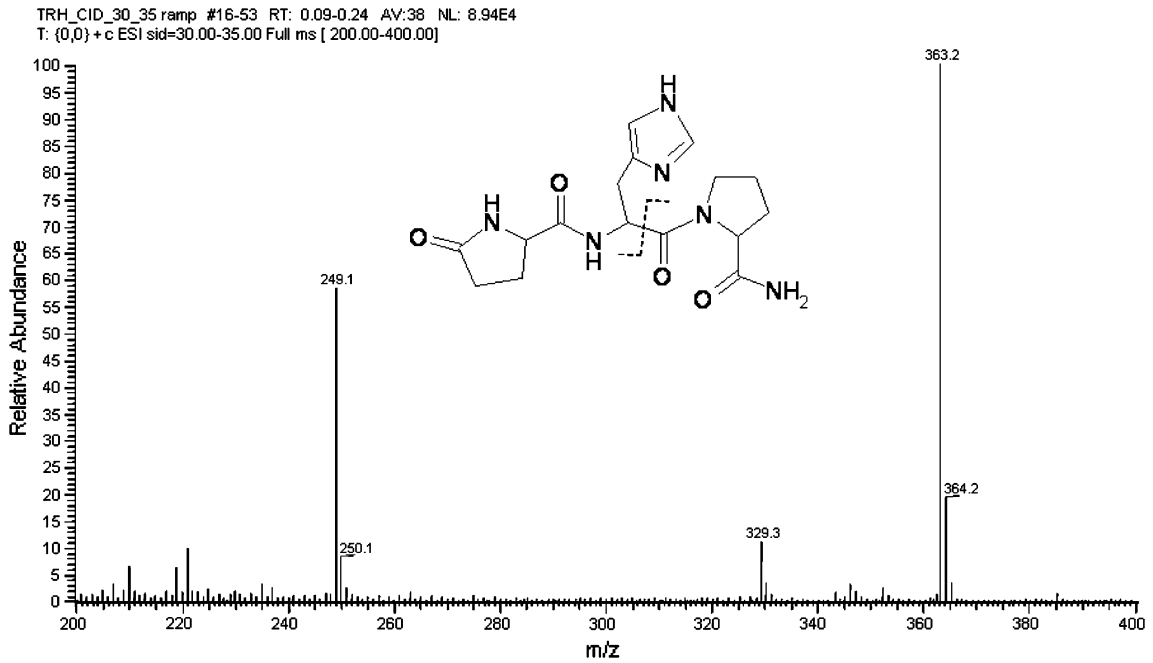

Fig. 4 Collision-induced dissociation (CID) spectrum of the TRH performed on the AQA single quadrupole mass spectrometer. A cone voltage ramp of 30–35 V was applied to induce fragmentation. The fragment ion at *m/z* 249.1 of the b_2 ion of <Glu–His is clearly visible in the fragmentation spectrum, along with the parent ion of TRH

calibration curve was determined in the concentration range between 250 and 1,000 fmol. Also in this case, the calibration curve showed an optimal linear response with a correlation coefficient of 0.999 (Fig. 6B). Analyses of aliquots of the hypothalamic extracts in duplicate by this method (Fig. 7) showed that TRH was 0.30 ± 0.07 pmol/mg (average value in four independent determinations on two rats), which is consistent with that found in SIM mode.

Discussion

Thyrotropin-releasing hormone (TRH), whose discovery by the group of Guillemin dates back to 1970, is engaged in the regulation of pituitary hormone release (Persani 1998). From its first characterisation, the interest on this small tripeptide has been continuously renewed by several evidences suggesting additional and unexpected roles for

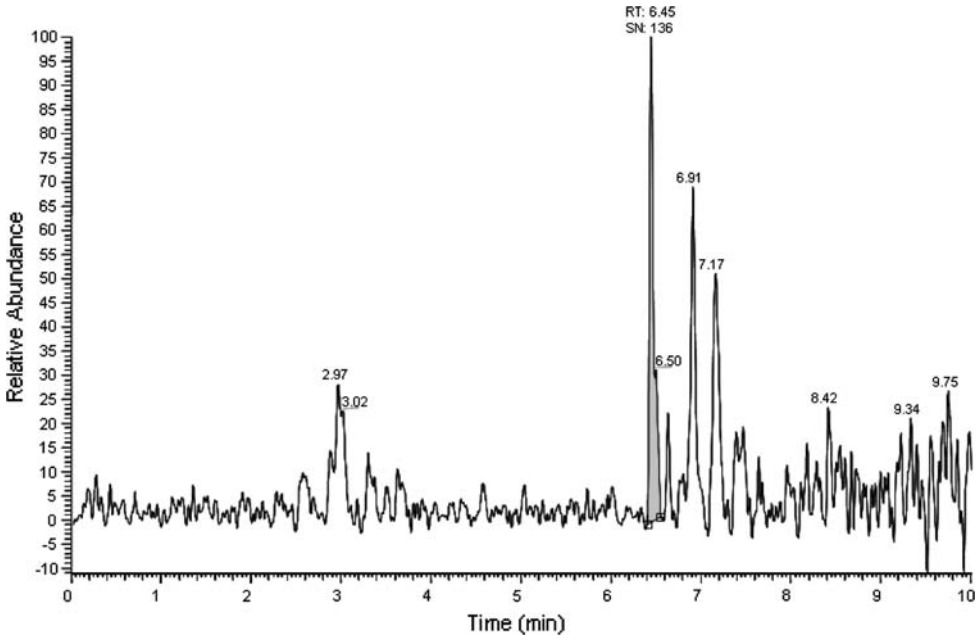

Fig. 5 Representative BPI chromatogram acquired in SIM mode of a rat hypothalamus extract. A peak at the elution time of TRH (*grey* in the chromatogram) was clearly detected with a signal to noise ratio of 136

Table 2 Content of TRH in rat hypothalami. For TRH quantification the average calculated amounts were multiplied for dilution factor (1:100) and for theoretical TRH molecular weight (362.2 Da)

	Hypothalamus weight (mg)	Average area (±SD)	Average amount (fmol) (±SD)	Nanograms (±SD)	Nanograms/mg (±SD)
Rat #1	40	13,066 ± 63	97.6 ± 0.6	3.5 ± 0.022	0.088 ± 0.0005
Rat #2	42	11,135 ± 149	79.3 ± 1.4	2.9 ± 0.051	0.072 ± 0.001

TRH, besides a well-established releasing activity supported by its extra-hypothalamic occurrence.

Furthermore, there is yet a growing interest in TRH for its altered levels in several tumours, including adenomas and melanomas (Garcia et al. 2000; Badiu et al. 2001; Ellerhorst et al. 2004). It has also been reported that TRH has a role in the regulation of carbohydrate metabolism in pancreas. Indeed, by preserving pancreatic islet cell function, a potential role of TRH in the treatment of diabetes mellitus has also been proposed (Luo and Jackson 2007; Luo et al. 2008). Moreover, recent findings report the activation of the extracellular signal-regulated kinase (ERK) and induction of mitogen-activated protein kinase phosphatase 1 (MKP-1) pathway by TRH stimulation in rat pituitary cells (Oride et al. 2008).

Collectively, these findings strongly encourage the development of specific and sensitive methods for TRH detection and quantification. Despite the availability of RIA and molecular biology techniques, the lack of molecular specificity of the detection still prevents an accurate and selective quantification of TRH. In addition, mRNA levels not always reflect the real gene expression, hence, there is still a need for complementary techniques for the univocal detection and quantification of TRH and TRH analogues. Importantly, hormone quantification within tissues provides useful information for understanding their biological functions.

High selective and sensitive MS-based methods for the measurement of endogenous or exogenous bioactive peptides in biological fluids have been previously reported for the determination of rhodopsin, of prostate-specific antigen (PSA), and C-reactive protein (Barnidge et al. 2003, 2004; Kuhn et al. 2004). Most of these applications involve the analysis of bioactive peptides in plasma or serum. In contrast, peptide analysis in tissues has been reported for determining endogenous neuropeptides, such as β-endorphin in the human pituitary glands (Dass et al. 1991). In a recent review on the identification and quantification of endogenous peptides in neuroendocrine tissues, Fricker et al. provided valuable information on the difficulties faced with the analysis of endogenous neuropeptides in neuroendocrine tissues (Fricker et al. 2006).

The interest in TRH and in its stable analogues is continuously increasing due to their therapeutic potential in treating several CNS and metabolical diseases. However, TRH identification and quantification within tissues under several conditions still remain a difficult task. It could indeed be useful to determine hormone dosage under physiological and pathological as well as following various pharmacological treatments. Mass spectrometry

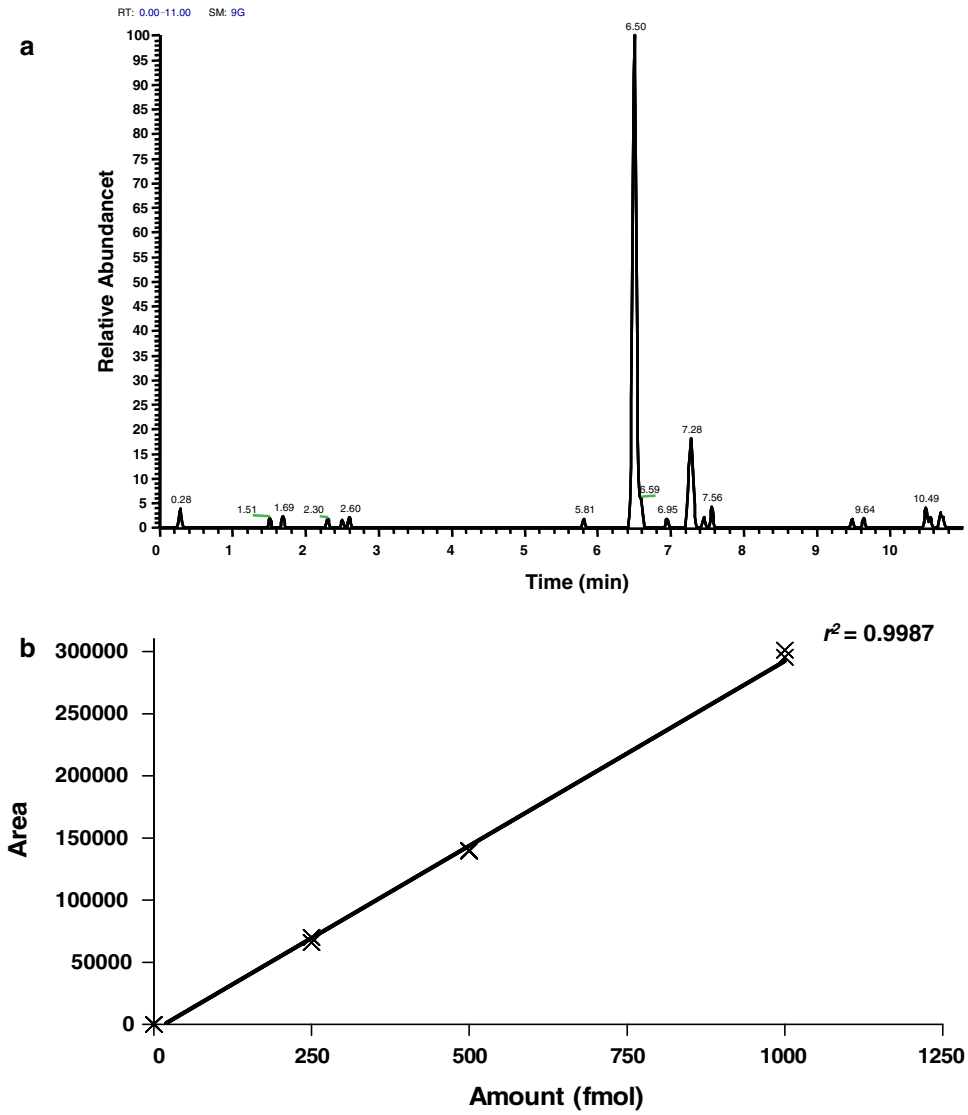

Fig. 6 **a** Representative BPI chromatogram acquired in SRM mode of TRH standard (200 fmol). Gaussian smoothing and baseline subtraction processing were applied on raw data. **b** Calibration curve deriving from the SRM acquisition mode. A linear polynomial type curve was used for data fitting

approaches are widely recognised as suitable techniques for protein and small molecule analysis. In particular, peptide quantification by liquid chromatography coupled with MS has proved to be very advantageous, due to the well-established selectivity and sensitivity of MS techniques. Nevertheless, for the quantification of biologically active peptides, immunological techniques are still widely employed, despite their limits in specificity. In the case of TRH, for example, discriminating immunoreaction signals deriving by closely related analogues often differing by only one out of three amino acids could be a very challenging task.

In this paper, a rapid and sensitive method for TRH quantification based on an MS approach in SIM as well as in SRM mode was developed. We have complemented the high sensitivity of SIM with the SRM to increase the molecular specificity. Noticeably, TRH gives rise, upon fragmentation, to the diagnostic b2 product ion at *m/z* 249.1. The identification and quantification of TRH from peptide extracts of rat hypothalami were also performed to test the suitability of the methodology for the detection within very complex biological samples. The concurrent enhancement of sensitivity and specificity by SIM and SRM acquisition modes greatly reduces the negative effects of interfering components from the biological matrix, yielding improved S/N ratios and lowering considerably the limits of detection. Importantly, the proposed methodology is applicable to the determination of TRH analogues with known amino acid sequence, with little modifications for the setting up of proper experimental parameters. Furthermore, the optimised LC–MS method described in the present work can be easily applied to even more sensitive mass spectrometers (e.g. triple quadrupole, linear traps, etc.) to further reduce the detection limits.

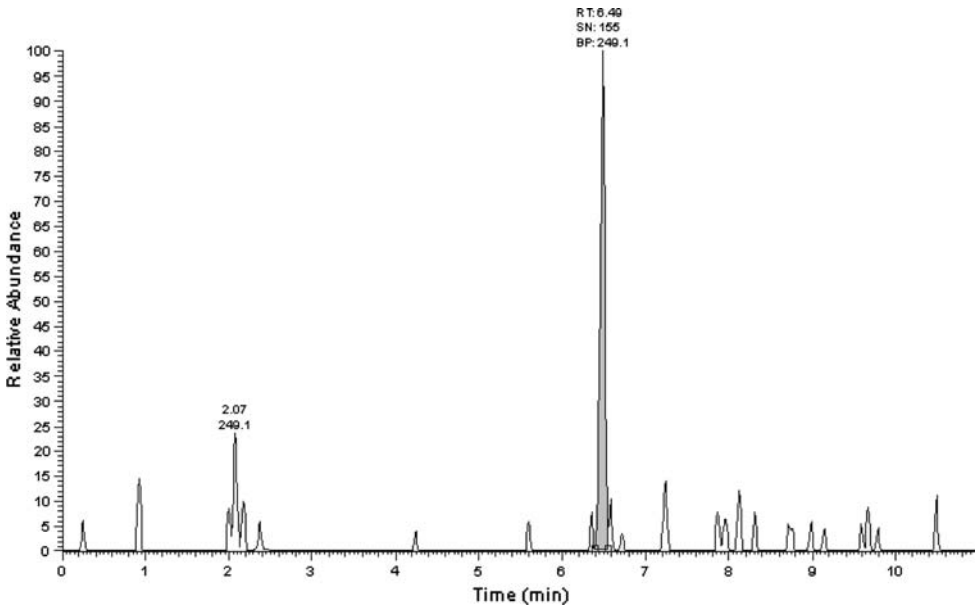

Fig. 7 Representative BPI chromatogram acquired in SRM mode of a rat hypothalamus extract. A very clean chromatogram was obtained with a predominant peak at the elution time of TRH, with a signal-to-noise ratio of 155

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