Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1071 (2005) 179–184
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Online preconcentration of thyrotropin-releasing hormone (TRH) by SDS-modified reversed phase column for microbore and capillary high-performance liquid chromatography (HPLC)

Rong Meng[a], Wanlin Xia[a], Mats Sandberg[b], Robert Stephens[c], Stephen G. Weber[a,*]

[a] *Department of Chemistry, University of Pittsburgh, 603 Chevron Science Center, 219 Parkman Drive, Pittsburgh, PA 15260, USA*
[b] *Department of Cell Biology, University of Gothenburg, Gothenburg, Sweden*
[c] *Department of Physiology, College of Medicine, The Ohio State University, Columbus, OH 43210, USA*

Available online 6 January 2005

Abstract

Thyrotropin-releasing hormone (TRH, pGlu-His-Pro-amide) is an important tripeptide existing in biological systems at low concentrations. It is a fairly hydrophilic peptide, cationic in acidic solutions. Preconcentration online before reversed phase chromatography separation can enhance concentration detection limits of hydrophobic, but not hydrophilic species. The hydrophilic TRH can be preconcentrated using a reversed phase precolumn charged with sodium dodecyl sulfate (SDS). The separation also uses SDS. The preconcentration is effective for a microbore system, achieving detection limit of 250 pM for a sample size of 500 μl with electrochemical detection of the biuret complex formed post column. Preconcentration using an online precolumn is also effective in packed capillary high-performance liquid chromatography (HPLC) with a detection limit of 3 nM in 24 μl.

Keywords: Online preconcentration; HPLC; Thyrotropin-releasing hormone; Capillary; Ion-pair

1. Introduction

Thyrotropin-releasing hormone (TRH) is an important neuropeptide originally discovered in the hypothalamus as a releasing factor for pituitary hormones such as thyrotrophin and prolactin [1,2]. From a therapeutic perspective, administration of TRH and some of its stable analogs, appear to have neuroprotective and anti-convulsive effects [3–8]. It is also present in other brain areas with proposed additional, although not yet fully elucidated, functions [9–11].

Radioimmunoassay (RIA) has been used for TRH assays in serum and various tissues from rat [12–16]. Though powerful, RIA suffers from the drawbacks that it is too selective to be useful for determining all the members in a family of related peptides, but may not be selective enough to determine a single peptide accurately. In fact, analogs of TRH that displayed similar immunoactivity as TRH have been found in various rat tissues [17–21]. Therefore, a "separate-and-detect" approach is preferred. High-performance liquid chromatography (HPLC) with UV–vis detection only works at low sensitivity [22–25]. While fluorescence detection following HPLC or CZE separations is a powerful technique for the determination of amino acids and peptides, it typically requires a primary amine or thiol group in the targeted molecule to be derivatized. Neither of these two functional groups exists in TRH. Mass spectrometry (MS) coupled to chromatography has been used qualitatively in characterizing this peptide [20] and to determine the sequence of some of its analogs that may not be distinguishable in RIA [21,26,27]. There are very few works on quantification of TRH by MS and these are not routine methods. Reports were found on field-desorption ionization following LC [28] and on fast atom bombardment ionization following GC by looking at the fragments [29]. A simpler detection method with high sensitivity and selectivity is still desirable.

* Corresponding author. Tel.: +1 412 624 8520; fax: +1 412 624 1668.
E-mail address: sweber@pitt.edu (S.G. Weber).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.12.032

Selective chromatographic detection of peptides has been established based on the reversible electrochemistry of the Cu(II)/Cu(III) couple in polydentate peptide complexes [30–33]. The detection relies on the coordination reaction of peptides and Cu(II), also called the biuret reaction [34,35]. TRH forms electroactive complex with Cu(II) thus is detectable with the above method. In rat brain, TRH concentration ranges from roughly 2 nM (cerebellum) to 350 nM (hypothalamus) [11]. The targeted sample for dialysate analysis—extracellular fluid, is expected to contain lower concentrations of TRH. The low endogenous levels in biological samples are a challenge for the electrochemical detection of bioactive peptides. One method to improve sensitivity is to preconcentrate the analytes. Moreover, on-line preconcentration is preferred over off-line for higher efficiency.

Works on online preconcentration methods for liquid separations are limited, especially for capillary LC. For hydrophobic analytes (e.g. hydrophobic peptides), reversed phase precolumns as injection loops trap and preconcentrate the analytes [36,37]. A simpler alternative is to inject a large volume of sample in a lower strength solvent, and elute the retained analytes with the higher strength mobile phase [38–40]. This method evidently will not apply to hydrophilic molecules. TRH is a hydrophilic cation in weak acidic solution, while the general mobile phases for peptide separation have acidic pH due to trifluoroacetic acid. This makes preconcentration on the reversed-phase column head not very promising. For charged analytes, a few works about strong ion exchange precolumns have been reported. In an analysis of trypsin digested BSA, strong cationic peptide fragments which featured Arg or Lys as terminal residues were trapped in a 250 μm i.d. strong ion exchange precolumn and eluted by a neutral buffer prior to a microbore HPLC system [41]. In the present work, we describe an online preconcentration method to enrich TRH through sorption in the presence of a surfactant. Ionic interactions have been widely used in liquid chromatography to achieve better separation on reversed phase columns [42,43]. To the best of our knowledge, no works have been reported on surfactant-assisted online preconcentration of cationic peptides.

2. Experimental

2.1. Microbore HPLC

Reagents and sources were as follows: sodium cabonate, sodium bicarbonate, cupric sulfate pentahydrate, disodium tartrate dihydrate, and sodium dodecyl sulfate (SDS) were from Sigma (St. Louis, MO, USA) and used without further purification. TFA was from Avocado (Heysham, Lancs, UK). TRH was from Sigma and stored at −20 °C. Doubly deionized water used in all solutions was obtained through a Milli-Q system (Millipore, Bedford, MA, USA). All other common reagents were of AR grade or better and purchased from commercial sources.

The chromatographic apparatus included a LC-10AD Shimadzu pump (Kyoto, Japan) delivering mobile phase (0.1% TFA, 2% 1-propanol, 20% acetonitrile and 3 mM SDS in water), a Rheodyne injector, and a Jupiter 150 mm × 1.0 mm C_{18} reversed phase column from Phenomenex (Torrance, CA, USA). The postcolumn biruet reagent (0.5 mM CuSO4, 3 mM sodium tartrate, and 1 M carbonate, pH 9.8) was delivered by a 100 DM syringe pump from ISCO (Lincoln, NE, USA). The typical flow rates were 50 μl/min in the column and 20 μl/min for the biuret reagent. A mixing tee and teflon tubing with 100 μm i.d. and 12.5 μl volume (both from Upchurch, Oak Harbor, WA, USA) were used as the postcolumn reactor. Both the column and the reactor were put in a temperature controller (model LC-23A, BAS, W. Lafayette, IN, USA) which was maintained at 50 °C.

A 4.0 mm × 2.0 mm C_{18} guard column from Phenomenex was used as the preconcentration column (also called precolumn or preconcentration loop in this work). It was connected to the injector by a 16 cm 62 μm i.d. PEEK (polyetheretherketone) tube (about 0.5 μl). Before each loading, the precolumn was preconditioned with 1250 μl of 0.1% TFA and 3 mM SDS solution. Then about 500 μl of analyte solution were loaded into the precolumn. There was a 2 min or longer delay time before the injector was switched to injection position.

The electrochemical detector was a BAS LC-4C thin layer flow cell, with a dual glassy carbon electrode (3.0 mm diameter) and a 13 μm thick teflon spacer (both from BAS). Detection potential was +0.9 V at the upstream electrode, + 0.1 V at the downstream electrode versus Ag/AgCl reference electrode (3 M NaCl, BAS). Data were collected and processed with EZChrom software (Sci. Software, San Ramon, CA, USA). All necessary fittings and tubings are from Upchurch (Oak Harbor, WA, USA).

2.2. Capillary HPLC

Reagents and sources were as follows: TFA and 1-propanol were from Sigma; disodium tartrate dihydrate and sodium hydroxide were from Baker (Phillipsburgh, NJ, USA); copper sulfate pentahydrate was from Fisher (Pittsburgh, PA, USA); acetonitrile (ACN), sodium carbonate, and sodium bicarbonate were from EM Science (Gibbstown, NJ, USA); TRH was from Bachem (King of Prussia, PA, USA); SDS was from Fluka (Switzerland). The copper sulfate pentahydrate and disodium tartrate dihydrate were recrystallized once from water. All other reagents were used without further purification, but purities were taken into account in preparation. De-ionized water from a Millipore A10 Synthesis system was used for all solutions. All mobile phases and biuret reagent solutions were filtered through 0.45 mm nylon membranes (Osmonics, Minnetonka, MN, USA) before use.

The chromatographic instrumentation included a Waters 600 E quaternary pump for mobile phase (0.1% TFA, 3% 1-propanol, 20% acetonitrile, and 2.2 mM SDS), a split tee for low flow rates, a VICI injector (Valco Instrument Company, Houston, TX, USA), and a home-packed capillary column.

Injections were made through 100 μm silica capillary with an Upchurch inline filter (part # M-520) to one of the injector ports. A Harvard Model 11 syringe pump (Harvard Apparatus, Inc., Holliston, MA, USA) was used to pump the biuret reagent (2.5 mM $CuSO_4$, 12.0 mM sodium tartrate, and 0.6 M carbonate, pH 9.83). Typical flow rates were 800 nl/min for the HPLC and 250 nl/min for the biuret. The two streams were mixed for postcolumn reaction in a home-made device [44]. At the end of the reactor, the copper(II)-peptide complexes were detected at +0.80 V versus Ag/AgCl (3 M NaCl) with a 10 μm carbon fiber electrode 1.0 mm in length. The potential was controlled with a BAS Epsilon potentiostat.

Capillary columns were slurry packed with the technique described previously [45]. Typically 100 μm silica capillaries were slurry packed with 2.0 μm prototype bridged hybrid C_{18} (Waters, Milford, MA, USA) reversed-phase particles. The packed length was typically 6–8 cm. The preconcentration loop was packed in a 7.0 cm long 75 μm i.d. silica capillary with 5 μm C_{18} packing material. The packed length was 2 mm. Before each loading it was preconditioned with a solution containing 0.1% TFA and 3.0 mM SDS. Reactors were constructed with 50 μm silica capillaries and 18 μm tungsten wires, according to previously published procedures [44]. The mixing length was 6 or 6.6 cm, stated in figure captions.

All fused-silica capillaries were from Polymicro (Phoenix, AZ, USA). All necessary fittings and tubings are from Upchurch. All syringes used are from Hamilton (Reno, NV, USA).

2.3. Brain tissue samples

The tissue samples were supplied by Prof. Mats Sandberg. The detailed preparation procedures have been previously described [43,46,47]. Briefly, rat hippocampus is homogenized and extracted with 90% (v/v) methanol. Lyophilized extracts are stored frozen until use. Before injection, samples were reconstituted in 500 μl water, sonicated 6 min, centrifuged for 10 min (Jouan, Winchester VA, 12,000 rpm), and filtered (0.2 μm). Typically, somewhat less than 500 μl are recovered for injection.

3. Results and discussion

In the dual electrode detection mode, the downstream (cathode) electrode typically has a better baseline and signal to noise ratio than the upstream (anode) electrode. We will focus our discussion on the downstream electrode signals in microbore HPLC.

As the postcolumn reaction is essential in the detection scheme, the mixer had to be optimized for complete reaction yet minimal band broadening. Preferably, the radial diffusion in the tubing needs to be predominant over axial diffusion and convection. We will call this regime of convective dispersion the Taylor regime [44,48]. It allows for good mixing when two flow streams come into one open tube without excessive band spreading. For microbore HPLC, a combine-and-split reactor achieved good mixing of eluents and biuret reagent. Fig. 1 shows a sketch of this reactor. It operates in the Taylor regime under the employed flow rate of 70 μl/min. Because of the three crosses employed, the reactor provides additional mixing over that in a single open tube with the same volume. The total reaction volume was 12.5 μl. This reactor was used throughout the microbore HPLC work. In the capillary HPLC experiments, staying in the Taylor regime, efficient mixing took place in a 120 nl capillary (50 μm i.d., 6.0 cm).

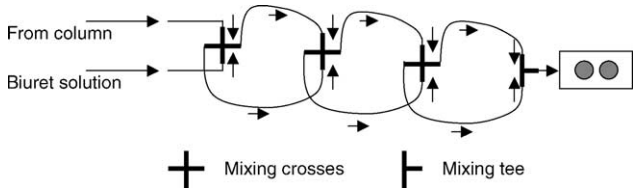

Fig. 1. Sketch of the combine-split reactor. The tubings connecting the crosses were 65 μm i.d., 40 cm long each. Crosses and the Tee have 0.05 cm through holes. Dimensions of the detection cell are in Section 2.

TRH is not retained in a typical TFA/ACN mobile phase (data not shown). This is due to the low molecular weight and high polarity of TRH. A hydrophobic anionic additive, heptafluorobutyric acid (HFBA), is able to shift the retention of TRH to longer times. Unfortunately, it was found that the added HFBA deactivated the electrode very quickly, making the detection irreproducible.

Alternatively, 2–3 mM SDS retained TRH as an additive in the mobile phase. Acetonitrile had to be included in the mobile phase to achieve a practical retention time. Fig. 2 shows the signal and retention time of TRH standard at various fractions of ACN in the SDS-containing mobile phase. In a 10% ACN mobile phase, TRH was not eluted within 40 min. Higher ACN level of 20% brought the TRH k' to a reasonable value of 2–3. In capillary HPLC, this percentage of ACN gives a similar k' value.

Under the above conditions, TRH displayed a linear response in the range of 100–500 nM. The regression of peak area (in nC) versus TRH mass (in pmol) gave an intercept much smaller than its uncertainty. Regression with the calibration curve forced through zero yields $y = 0.22x$

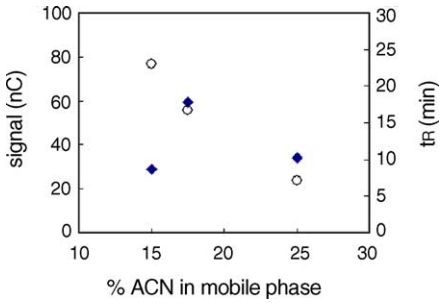

Fig. 2. Optimizing acetonitrile content in the SDS-containing mobile phase. Mobile phase contained 0.1% TFA, 2% 1-propanol, 3.0 mM SDS, and stated amount of ACN. TRH concentration: 500 nM. Sample loop: 20 μl; ♦: peak area; ◯: retention time.

(R^2 = 0.9845). The detection limit (3σ) is 4.8 nM. (S/N value for 100 nM injection was about 55, for 500 nM injection was about 400).

In an effort to push the concentration detection limit to a lower level, we attempted to use the column head to preconcentrate TRH by analogy to reversed phase LC. However, because the large volume of low strength solvent (usually water) disturbed the equilibrium of SDS between the mobile and stationary phases, a long equilibration time, at least several hours, was found necessary to get reproducible separation.

A postulated solution to this problem is to put a small C_{18} precolumn in the injection loop, which is preconditioned with SDS-containing aqueous solution. The SDS generates a negatively charged layer on the stationary phase by hydrophobic interaction. When the sample prepared in aqueous solution is loaded into the preconditioned precolumn, molecules partition between the "mobile phase" water and the SDS-modified stationary phase. During this procedure the anionic and neutral hydrophilic and weakly hydrophobic species go through the precolumn to waste, while the cations and hydrophobic species stay in the precolumn. When the loop is switched into the separation system, the mobile phase desorbs the TRH (and some SDS). One of the advantages of this strategy is that it removes the interfering species such as salts which commonly exist in biologic samples.

It is interesting to find that the solvent composition of sample has a great effect on the preconcentration. Shown in Fig. 3, TRH in water gave a much narrower peak than in 0.1% TFA/3 mM SDS (the precondition solution). This implies that biological samples such as microdialysates could be directly introduced to the precolumn.

As a demonstration of the preconcentration approach, 500 μl of TRH standard solutions from 5 to 100 nM were loaded each time and detected. In Fig. 4 the chromatograms show that both the retention time and peak width of TRH are consistent. Similar to the case without preconcentration, the intercept from regreassion (nC versus pmol) was also smaller than the associated standard error. Regression anew gave a calibration curve of $y = 143.1x$ (R^2 = 0.9490). The detection limits (3σ in each case) from chromatograms of 5, 10 and 20 nM injections were 142, 340 and 250 pM. We conclude that 250 pM is the detection limit of this technique for TRH.

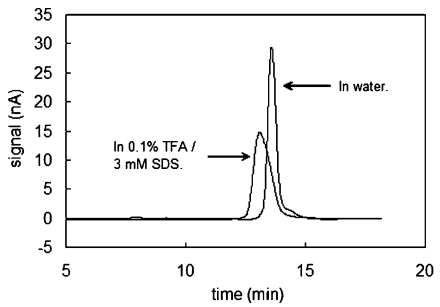

Fig. 3. Comparison of signals of preconcentrated TRH prepared in SDS-containing solution and in water. TRH concentration: 500 nM; loaded volume: 500 μl.

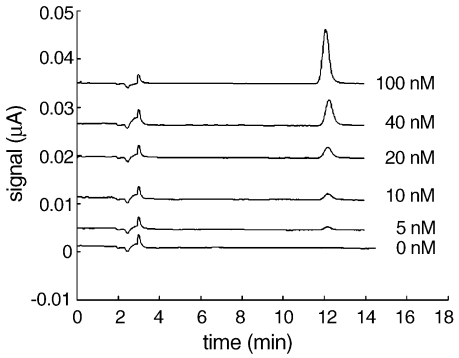

Fig. 4. Signals of preconcentrated TRH at different concentrations in microbore HPLC.

The low detection limit obtained from this detection approach made analysis in biological samples promising. Homogenized rat brain tissue was then introduced into the set-up. In Fig. 5, a clear peak is observed at the same retention time that TRH is eluted. It corresponds to 0.3 pmol TRH per milligram of hippocampus tissue. This value was within the concentration range from the literature [11,49,50]. The chromatogram is fairly clean because the preconcentration step removed many interfering species.

It could be concluded that the above preconcentration method was successful in terms of detection limit and compatibility with biological samples. Sensitivity was enough for physiological analysis, and selectivity was assured by the use of precolumn and dual electrode cell. However, there is a very serious drawback, which is the large sample size required for the determination. The loaded volume in the brain tissue analysis was 470 μl. This is not practical for many samples. In this perspective, the system volume had to be decreased to accommodate much smaller sample sizes. Accordingly, the preconcentration strategy had to be carried out in a capillary format. The chemistry behind it should work regardless of the size of the column.

In a capillary chromatography systems there are two potential sources of extracolumn band spreading: precolumns and post column reactors. A locally developed post column reactor [44] has been demonstrated to meet the requirement

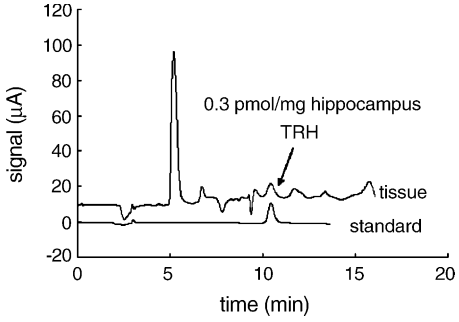

Fig. 5. TRH determination in a homogenized rat brain tissue sample by microbore HPLC. Sample was reconstituted in 500 μl water, sonicated, centrifuged and filtered. A volume of 470 μl was injected. The standard was 100 nM TRH.

of low sample volume and virtually no band broadening post column.

As for the precolumn, there are no commercial guard columns to use as the precolumn to match the much smaller size of the separation column. In preparing precolumns with capillaries, not surprisingly, the system brought up some engineering issues. The most important concern was the void volume of the tubing that connects the precolumn to the injector relative to the volume of the precolumn. For uniform sample loading, the void volume should be negligible compared to that of the precolumn. However, it is extremely hard to achieve because the packed precolumn will already be very small. At the same time, the port-to-port distance of the injector determines the minimum length that can be used. If the capillary loop/precolum is too short, the strain will lead to breakage. Larger i.d. capillaries, though providing lower back pressure and therefore allowing for easy manual injection, are easily broken at the frit. In order to reduce the unpacked void volume, shorter, smaller i.d. capillaries are preferred. Loop/precolumns that failed (broke at the frit) were (o.d./i.d., μm) 360/250, 330/180, and a system that was 360/40 in series with a <1 cm packed 360/250 and another 360/40. In the latter system, a single high pressure connector (LC Packings TF-250) covered the precolumn and overlapped with the 360/40 on both sides. On the other hand, loop/precolumns with thicker walls, 360/75, were successful. For 75 μm i.d. silica capillary, a 7 cm total length preconcentration loop did not break on being connected to the injector. Only a 2 mm length at the end of this capillary was packed. The back pressure was low enough to be permit injection with a 50 μl Hamilton gastight syringe.

To test if such a packed preconcentration loop works, TRH standard solutions of the same concentration were loaded into the loop at different loading volumes. Loaded volumes were corrected for the volume of the tubing from injection port to injector and the dead volume of an inline filter (1.5 μl total). After each loading, the loaded loop was switched into the system for analysis. The signals from Cu(II)-TRH were proportional to the loaded volume as expected, with a rather good correlation coefficient (see Fig. 6). The bands were tailing to some extent, but the tailing did not get worse as loaded volume increased. This implied that the band shape was not affected by overloading of the precolumn, but by other parameters, most likely the construction of the precolumn.

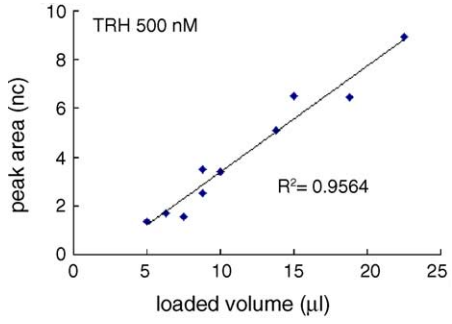

Fig. 6. Peak areas resulting from different loaded volumes of 500 nM TRH standards with preconcentration in capillary HPLC.

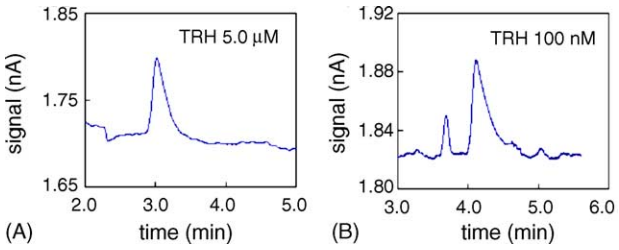

Fig. 7. Comparison of TRH signals before and after preconcentration in capillary HPLC. The postcolumn mixing length was 6.0 cm in 7a and 6.6 cm in 7b.

Fig. 7 gives a comparison of the TRH signal before and after the preconcentration column was introduced. The 5 μM and 100 nM TRH standards gave similar signal to noise ratio (approximately 100 in both cases). Fig. 7b signal corresponds to a detection limit of 3 nM. It can be concluded that after preconcentration the concentration sensitivity of the current detector to TRH could be increased up to 50 fold.

Injection to injection repeatability is a function of the amount injected for the microbore experiments. Coefficients of variation range from 3% for 5 nM (500 μl) injected to 21% for 100 nM (500 μl) injected. For the capillary system, the coefficient of variation is about 20% for volumes of 8.5 and 13.5 μl (500 nM).

The precolumn can be improved by optimizing its construction. The packed length should not be too short, in that sufficient partition process was required to stack TRH molecules into the precolumn. On the other hand, the packed length could not be too long, otherwise the back pressure introduced in loading the precolumn would be hard to handle. Large i.d. tubings can be used for larger packed volumes, if ways can be found to prevent the thinner-wall capillaries from snapping. To limit void volume is also very important. Currently, the volume ratio of the precolumn and the separation column is much smaller than that in the microbore HPLC. This is mostly due to the engineering restrictions associated with compromising between better mechanical strength as well as smaller void volume and bigger precolumn capacity.

4. Conclusion

SDS-modified precolumn as a preconcentration sample loop is successful. The same chemistry worked on both microbore and capillary HPLC systems. Introducing of preconcentration column improved the detection limit of TRH 30–50 fold in both systems. Analysis of biological samples, especially microdialysates, is ultimate in the study of neuropeptides. The capillary HPLC system has to be improved for this purpose. Two feasible ways are improvement of the precolumn construction and improvement of the electrochemical detector.

Acknowledgements

The authors are grateful to the NIH for funding through grant GM44842. We are also grateful to Dr. Ed Bouvier (Waters Corp.) for providing us with the packing materials.

References

[1] J. Boler, F. Enzmann, K. Folkers, C.Y. Bowers, A.V. Schally, Biochem. Biophys. Res. Commun. 37 (1969) 705.
[2] C.Y. Bowers, H.G. Friesen, P. Hwang, H.J. Guyda, K. Folkers, Biochem. Biophys. Res. Commun. 45 (1971) 1033.
[3] A.I. Faden, Arch. Neurol. 43 (1986) 501.
[4] A.J. Prange Jr., P.P. Lara, I.C. Wilson, L.B. Alltop, G.R. Breese, Lancet 2 (1972) 999.
[5] L. De Marinis, A. Mancini, D. Valley, A. Bianchi, R. Gentilella, I. Liberale, V. Mignani, M. Pennis, F. Della Corte, Clin. Endocrinol. 50 (1999) 741.
[6] S. Manaka, K. Sano, Neurosci. Lett. 8 (1978) 255.
[7] G. Stocca, A. Nistri, Neurosci. Lett. 184 (1995) 9.
[8] H. Ujihara, R.M. Xie, M. Sasa, K. Ishihara, Y. Fujita, M. Yoshimura, T. Kishimoto, T. Serikawa, J. Yamada, S. Takaori, Eur. J. Pharmacol. 196 (1991) 15.
[9] P. Kachidian, P. Poulat, L. Marlier, A. Privat, J. Neurosci. Res. 30 (1991) 521.
[10] K. Iverfeldt, P. Serfozo, L.D. Arnesto, T. Bartfai, Acta Physiol. Scand. 137 (1989) 63.
[11] A. Winokur, R.D. Utiger, Science 185 (1974) 265.
[12] Y. Fuse, D.H. Polk, R.W. Lam, D.A. Fisher, Endocrinology 127 (1990) 2501.
[13] H. Nagai, K. Morise, T. Mitsuma, A. Furusawa, H. Kaneko, K. Uchida, H. Yamamoto, J. Gastroenterol. 30 (1995) 142.
[14] E.A. Nillni, C. Vaslet, M. Harris, A. Hollenberg, C. Bjorbaek, J.S. Flier, J. Biol. Chem. 275 (2000) 36124.
[15] K. Mohari, G. Janoki, L. Korosi, Izotoptechnika Diagnosztika 34 (1991) 137.
[16] J. Leppaluoto, A.S. Suhonen, J. Clin. Endocrinol. Metabol. 54 (1982) 914.
[17] M.W. Ghilchik, M. Tobaruela, J. del Rio-Garcia, D.G. Smyth, Biochim. Biophys. Acta 1475 (2000) 55.
[18] P.J. Gkonos, C.K. Kwok, N.L. Block, B.A. Roos, Prostate (New York, NY, United States) 23 (1993) 135.
[19] P.J. Gkonos, C.K. Kwok, N.L. Block, B.A. Roos, Peptides (Tarrytown, New York) 15 (1994) 1281.
[20] E.S. Graham, C.A. Webster, D.G. Hazlerigg, P.J. Morgan, J. Neuroendocrinol. 14 (2002) 945.
[21] R. Bilek, Physiol. Res. (Prague) 49 (2000) s19.
[22] G.N.S. Rao, J.W. Sutherland, G.N. Menon, Pharmaceut. Res. 4 (1987) 38.
[23] A.H. Waterfall, R.W. Clarke, G.W. Bennet, Neurosci. Lett. 151 (1993) 97.
[24] W.J. Sheward, A.J. Harmar, H.M. Fraser, G. Fink, J. Chromatogr. 222 (1983) 381.
[25] E. Spindel, D. Pettibone, L. Fisher, J. Fernstrom, R. Wurtman, J. Chromatogr. 222 (1981) 381.
[26] B.W. Gibson, L. Poulter, D.H. Williams, J. Natl. Prod. 49 (1986) 26.
[27] D.B. Lackey, J. Biol. Chem. 267 (1992) 17508.
[28] D.M. Desiderio, J.L. Stein, M.D. Cunningham, J.Z. Sabbatini, J. Chromatogr. 195 (1980) 369.
[29] N. Heki, M. Noto, H. Hosojima, Nippon Naibunpi Gakkai Zasshi 53 (1977) 690.
[30] A.M. Warner, S.G. Weber, Anal. Chem. 61 (1989) 2664.
[31] J.-G. Chen, S.G. Weber, Anal. Chem. 67 (1995) 3596.
[32] H. Tsai, S.G. Weber, J. Chromatogr. 515 (1990) 451.
[33] S.J. Woltman, J.-G. Chen, S.G. Weber, J.O. Tolley, J. Pharmaceut. Biomed. Anal. 14 (1995) 155.
[34] D.W. Margerum, L.F. Wong, F.P. Bossu, K.L. Chellappa, J.J. Czarnecki, S.T. Kirksey Jr., T.A. Neubecker, Adv. Chem. Ser. 162 (1977) 281.
[35] Y. Noda, Bull. Chem. Soc. Jpn. 40 (1967) 1264.
[36] M. Frommberger, P. Schmitt-Kopplin, G. Ping, H. Frisch, M. Schmid, Y. Zhang, A. Hartmann, A. Kettrup, Anal. Bioanal. Chem. 378 (2004) 1014.
[37] Y. Shintani, X. Zhou, M. Furuno, H. Minakuchi, K. Nakanishi, J. Chromatogr. A 985 (2003) 351.
[38] H. Shen, S.R. Witowski, B.W. Boyd, R.T. Kennedy, Anal. Chem. 71 (1999) 989.
[39] I. German, M.G. Roper, S.P. Kalra, E. Rhinehart, R.T. Kennedy, Electrophoresis 22 (2001) 3659.
[40] G. Mitulovic, M. Smoluch, J.-P. Chervet, I. Steinmacher, A. Kungl, K. Mechtler, Anal. Bioanal. Chem. 376 (2003) 946.
[41] G. Zhang, H. Fan, C. Xu, H. Bao, P. Yang, Anal. Biochem. 313 (2003) 327.
[42] J.-G. Chen, S.G. Weber, L.L. Glavina, F.F. Cantwell, J. Chromatogr. A 656 (1993) 549.
[43] W. Xia, M. Sandberg, S.G. Weber, J. Chromatogr. B 705 (1998) 251.
[44] A.T. Beisler, E. Sahlin, K.E. Schaefer, S.G. Weber, Anal. Chem. 76 (2004) 639.
[45] R.T. Kennedy, J.W. Jorgenson, Anal. Chem. 61 (1989) 1128.
[46] J.R. Mohammed, T.A. Saska, J. Chi, R.L.J. Stephens, Brain Res. 695 (1995) 100.
[47] W. Xia, M. Sandberg, S.G. Weber, J. Pharmaceut. Biomed. Anal. 19 (1999) 261.
[48] R.F. Probstein, Physicochemical Hydrodynamics, John Wiley & Sons, New York, 1994.
[49] M. Mendez, P. Joseph-Bravo, M. Cisneros, M.A. Vargas, J.L. Charli, Peptides (New York, NY, United States) 8 (1987) 291.
[50] E.C. Griffiths, G.W. Bennett, Thyrotropin-Releasing Hormone, Raven Press, New York, 1983.