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Analysis of iodinated peptides by LC-DAD/ESI ion trap mass spectrometry

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Abstract

The analysis of iodinated peptides resulting from chloramine-T (CAT), Iodo-Beads®, Iodo-Gen® and lactoperoxidase iodination reactions in the preparation of nanomole quantities ^{125}I and ^{123}I labelled tracers is described. Seven different model peptides were evaluated, varying in molecular weight from 294 (LY-dipeptide) to 2518 (obestatin containing 23 amino acid residues). Two different RP-C_{18} columns were used, each with a different gradient system based on aqueous formic acid and acetonitrile. Electrospray ionization (ESI) ion trap mass spectrometry was used for identification of the chromatographic eluting components of the reaction mixtures, while UV (DAD) served quantitative purposes. Non-, mono-, di-, tri- and tetra-iodinated peptides (respectively NIP, MIP, DIP, 3IP and 4IP) eluted in that order and were well separated from each other. An empirical model was derived. The applicability of this approach was demonstrated by the analysis of different reaction mixtures.

Keywords: Peptide iodination; LC-DAD/MS; Radio-HPLC; Obestatin; Neurotensin; Enkephalin; GGYR; LLY; VYV; LY

1. Introduction

Since the identification of secretin by Bayliss and Starling as a hormone secreted principally by the duodenal mucosa [1], over hundreds of biologically active peptides have been discovered. These compounds exhibit a wide range of actions, and have been proven useful in the field of medicine. However, numerous bioactive peptides are still to be identified and characterized. As RP-HPLC is capable of separating peptides, it has become a well-established method for the characterization, analysis and purification of these biomolecules [2–5].

Peptide radioiodination (*i.e.* incorporation of radioactive iodine such as ^{123}I, ^{125}I or ^{131}I into a peptide) is a technique commonly used for *in vitro* radioligand investigations as well as for medical imaging and therapy. Several direct and indirect iodination procedures currently exist. The most widely used direct labelling techniques are based on radioiodination of tyrosine and histidine amino acid residues with chloramine-T (CAT) [6], Iodo-Gen® (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) [7], lactoperoxidase [8], and the related solid-state variants Iodo-Beads® [9] or Enzymobeads® [10]. An alternative method to direct iodination is the conjugation of the peptide with a small radioiodinated molecule (*syn.* prosthetic group) such as the Bolton-Hunter reagent (*N*-hydroxysuccinimide ester of 3-(4-hydroxyphenyl)propionic acid) [11]. These indirect labelling methods are used in case of absence of tyrosine and histidine residues or when these amino acids are necessary for the peptide activity.

Peptide radioiodination results in formation of several mono- and higher-radioiodinated peptides that may exhibit different biological activities [12] and/or pharmacokinetic properties. Differences are also observed with the non-iodinated parent peptide.

Until now, only a few analytical characterizations have been performed on iodinated peptides. Loot et al. [12] reported the HPLC analysis of iodinated angiotensin-(1-7) using chloramine-T. Clear separation between the non-iodinated peptide and the

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mono- and di-iodinated derivatives was obtained with peak identification by mass spectrometry. Crim et al. [13] described the HPLC analysis of two small related insect neuropeptides ^{125}I labelled using chloramine-T, Iodo-Gen® and lactoperoxidase. Woloszczuk et al. [14] reported chromatofocussing on synthetic calcitonin and Tyr(0)-katacalcin radioiodinated with Iodo-Gen®. To our best knowledge, analytical characterization of a series of iodinated model peptides with liquid chromatography diode array detection/mass spectrometry (LC-DAD/MS), that were produced with multiple direct labelling techniques, has not been published yet.

For the current study, 7 model peptides consisting of 2–23 amino acid residues were selected for comparison of above direct iodination techniques and LC-DAD/MS characterization: LY, VYV, LLY, GGYR, leucine enkephalin (YGGFL), neurotensin (pELYENKPRRPYIL) and mouse obestatin (FNAPFDVGIKLSGAQYQQHGRAL-NH_2). Both neurotensin and obestatin contain two aromatic amino acid residues in their sequence which are available for iodination: respectively two tyrosyl, and one tyrosyl plus one histidyl groups [15]. The largest of these selected peptides, obestatin, is a recently discovered appetite regulating peptide hormone [16] that receives much attention in view of the development of drugs for eating disorders such as obesity and anorexia nervosa.

2. Experimental

2.1. Reagents

Iodo-Beads® and pre-coated Iodo-Gen® tubes were purchased from Pierce (Rockford, IL, USA). Lactoperoxidase was purchased from Sigma (St. Louis, CA, USA). HPLC gradient grade acetonitrile was purchased from Rathburn (Walkerburn, UK). LC–MS grade formic acid was purchased from Fluka (Buchs, Switzerland). Na^{125}I was purchased from MDS Nordion (Fleurus, Belgium). Water was purified in the laboratory by distillation of demineralized water. All other reagents were purchased from Merck (Darmstadt, Germany).

2.2. Peptides

All peptides were purchased from Sigma (St. Louis, MO, USA), except for mouse obestatin which was obtained from California Peptide Research (Napa, CA, USA) (gift from KULeuven). The concentrations used were derived from the quality control results stated on the certificates of analysis issued by the manufacturers: peptide content by amino acid analysis, water content by Karl Fischer, acetate content and/or HPLC purity.

2.3. Peptide lyophilization

Prior to iodination, peptides were dissolved in 0.1% (m/v) aqueous trifluoroacetic acid (at about 1 mg/mL), dispensed into microtubes and lyophilized using a Lyovac GT4 pre-cooled shelve freeze-dryer (Leybold, Cologne, Germany).

2.4. Peptide iodination using chloramine-T

The following method is based on the procedure described by Hunter and Greenwood [6]. It is used for peptides containing no amino acid residues sensitive to the oxidizing or reducing agent such as methionine, cysteine and tryptophan [17,18].

To a solution containing 100 nmol of peptide in 100 μL of phosphate buffered saline (100 mM, pH 7.4), 30 respectively 15 μL of chloramine-T solution (200 respectively 100 nmol), and 20 respectively 10 μL of sodium iodide solution (150 respectively 75 nmol) were subsequently added. After 40 s at room temperature, 30 respectively 15 μL of sodium metabisulphite solution (4 mg/mL) and 420 respectively 460 μL of phosphate buffered saline (100 mM, pH 7.4) was added.

As only small amounts of neurotensin and obestatin were available, in these cases 10 nmol of peptide was iodinated with chloramine-T, sodium iodide and sodium metabisulphite solutions diluted at 10% (v/v).

Since neurotensin contains two tyrosine residues, 10 nmol of peptide was also reacted with 30 and 40 μL of sodium iodide solution (22.5 and 30 nmol, respectively) and chloramine-T and sodium metabisulphite amounts increased accordingly.

Since mouse obestatin contains one tyrosine and one histidine residue, 10 nmol of peptide was also reacted at pH 8.2 with 20 and 60 μL of sodium iodide solution (15 and 45 nmol, respectively) and chloramine-T and sodium metabisulphite amounts adjusted accordingly.

2.5. Peptide iodination using Iodo-Beads®

The procedure was based upon Markwell [9].

To 20 μL of sodium iodide solution (150 nmol), one previously rinsed bead was added, and allowed to react for 5 min at room temperature. A solution containing 100 nmol of peptide in 100 μL of phosphate buffered saline (100 mM, pH 7.4) was added, and the reaction was allowed to proceed for 15 min until 100 μL of solution was removed and diluted with 400 μL of phosphate buffered saline (100 mM, pH 7.4).

For neurotensin and obestatin, 10 nmol of peptide was iodinated with sodium iodide solutions diluted at 10% (v/v) (15 nmol) and all other parameters remaining unchanged.

2.6. Peptide iodination using Iodo-Gen®

The procedure was described by Salacinski et al. [7].

Twenty microliter of sodium iodide solution (150 nmol) was transferred into a previously rinsed pre-coated Iodo-Gen® tube containing 100 nmol of peptide in 100 μL of phosphate buffered saline (100 mM, pH 7.4). After 15 min at room temperature, the reaction was stopped by removing 100 μL of solution, and dilution with 400 μL of phosphate buffered saline (100 mM, pH 7.4).

For neurotensin and obestatin, 10 nmol of peptide was iodinated with sodium iodide solutions diluted at 10% (v/v) (15 nmol) and all other parameters remaining unchanged.

2.7. Peptide iodination using lactoperoxidase

Below method is based on the procedure described by Marchalonis [8].

To a solution containing 100 nmol of peptide in 100 μL of phosphate buffered saline (100 mM, pH 7.4), 20 μL of sodium iodide solution (150 nmol), 80 μL of lactoperoxidase solution (0.04 mg/mL) and 20 μL of hydrogen peroxide (0.012%, v/v) were subsequently added. After 10 min at room temperature, a second amount of hydrogen peroxide (20 μL, 0.012%, v/v) was added, and again allowed to react for 10 min at room temperature until 30 μL of sodium metabisulphite solution (4 mg/mL) and 330 μL of phosphate buffered saline (100 mM, pH 7.4) was added.

For neurotensin and obestatin, 10 nmol of peptide was iodinated with sodium iodide, lactoperoxidase, hydrogen peroxide and sodium metabisulphite solutions diluted at 10% (v/v) and all other parameters remaining unchanged.

2.8. Peptide radioiodination

As a demonstration of the applicability towards radioiodination of peptides, leucine enkephalin was labelled with ^{125}I using the carrier-added CAT technique.

To a solution containing 100 nmol of peptide in 100 μL of phosphate buffer (130 mM, pH 7.4), 30 μL of chloramine-T solution (125 nmol) and 20 μL of carrier-added ^{125}I solution (100 nmol NaI + 100 μCi ^{125}I) were subsequently added. After 40 s at room temperature, 30 μL of sodium metabisulphite solution (2.35 mg/mL) and 420 μL of phosphate buffer (130 mM, pH 7.4) was added.

2.9. Liquid chromatography instrumentation and conditions

The LC-UV/MS apparatus consisted of a Spectra System SN4000 interface, a Spectra System SCM1000 degasser, a Spectra System P1000XR pump, a Spectra System AS3000 autosampler and a Finnigan MAT LCQ mass spectrometer in positive ion mode (all Thermo, San Jose, CA, USA) equipped with a SPD-10A UV-VIS detector set at 215 nm (Shimadzu, Kyoto, Japan) and Xcalibur 1.2 software (Thermo) for data acquisition.

The HPLC-PDA apparatus consisted of a Waters Alliance 2695 separations module and Waters 2996 photodiode array detector equipped with Empower 2 software for data acquisition (all Waters, Milford, MA, USA).

The radio-HPLC apparatus consisted of a LaChrom Elite L-2130 pump with degasser, a LaChrom Elite L-2300 column oven, a LaChrom Elite L-2400 UV detector (all Hitachi, Tokyo, Japan), a Rheodyne 7725i manual injector with 20 μL sample loop (Rheodyne, Rohnert Park, CA, USA) and a Berthold LB500 HERM radioactivity detector (Berthold Technologies, Bad Wildbad, Germany) equipped with EZChrom Elite Version 3.1.7 software for data acquisition (Scientific Software, Pleasanton, CA, USA).

For the experiments described in this paper, the columns (250 × 4.6 mm i.d., 5 μm) used were: Alltima HP C_{18} AQ (Alltech, Deerfield, IL, USA) and Vydac P&P C_{18} (Grace Vydac, Hesperia, CA, USA). The column temperature was maintained at 30 °C.

The mobile phase consisted of: (A) 0.1% (v/v) formic acid in water and (B) 0.1% (v/v) formic acid in acetonitrile. Linear gradient elution was performed at a flow rate of 1.0 mL/min. A different gradient program (*i.e.* different slope) was applied for each peptide.

3. Results and discussion

3.1. Peptide iodination

All peptide iodination reactions resulted in significant formation of iodinated species, except for obestatin with Iodo-Beads® as oxidizing agent (Table 1). On average, iodination of the model peptides using solid phase oxidants (*i.e.* Iodo-Beads® and Iodo-Gen®) resulted in lower peptide recoveries ($84 \pm 17\%$, $n = 14$) compared to the chloramine-T or lactoperoxidase-H_2O_2 method ($99 \pm 8\%$, $n = 25$).

In the experiments performed as described above, the mean iodination yields (*i.e.* number of iodide atoms incorporated in the peptide versus sodium iodide available) were decreasing in following order: chloramine-T, Iodo-Gen®, lactoperoxidase and Iodo-Beads® (111%, 96%, 62% and 17%, respectively).

Under the conditions applied, the formation of mono-iodinated peptide at pH 7.4 is decreasing in the following order: lactoperoxidase, chloramine-T (with 0.75 mol equivalents of I^-), Iodo-Gen®, chloramine-T (with 1.5 mol equivalents of I^-) and Iodo-Beads® (42%, 38%, 23%, 18% and 11%, respectively).

It was observed that the formation of di-iodinated peptide at pH 7.4 decreased in the following order: chloramine-T (with 1.5 mol equivalents of I^-), Iodo-Gen®, lactoperoxidase, chloramine-T (with 0.75 mol equivalents of I^-) and Iodo-Beads® (73%, 60%, 27%, 23% and 7%, respectively).

For obestatin, it was observed that the chloramine-T technique with different pH could differentiate between the tyrosine and the histidine amino acid residues (*i.e.* tri- and tetra-iodinated peptides are formed at pH 8.2, and not at pH 7.4).

3.2. Spectral data

The MS spectra obtained with the peptides and the iodinated derivatives (Table 2) correspond to their respective molecular structures. A shift of $[M+H]^+$ ion = +126 successively between NIP, MIP, DIP, 3IP and 4IP was observed, corresponding to increasing iodination degrees. A typical MS spectrum for the MIP of obestatin is given in Fig. 1, with peaks at *m/z* 1321.6, 881.7 and 661.7 attributed to $[M+2H]^{2+}$, $[M+3H]^{3+}$ and $[M+4H]^{4+}$, respectively. As an example, a tandem MS spectrum obtained on mono-iodinated leucine enkephalin is given in Fig. 2 to demonstrate the applicability of MS/MS for identity confirmation purposes.

From the UV spectral data obtained on the single-tyrosine containing peptides (Table 2), the following mean bathochromic

Table 1
Iodination of model peptides

Model peptide	Amount (nmol)	Iodination procedure	NaI (nmol)	pH	Amount of oxidant	Peptide recovery (%)[a]	Area% (UV at 215 nm)[b]				
							NIP	MIP	DIP	3IP	4IP
Leu-Tyr	101.9	Chloramine-T	154.8	7.4	200 nmol	100	3	13	83		
			77.4		100 nmol	100	37	38	25		
		Iodo-Beads®	154.8		1 bead	86	73	16	11		
		Iodo-Gen®	154.8		1 tube	101	23	14	63		
		Lactoperoxidase	154.8		4.8×10^{-3} µL H_2O_2	100	39	38	23		
Leu-Leu-Tyr	99.7	Chloramine-T	154.8	7.4	200 nmol	98	0	0	100		
			77.4		100 nmol	100	22	43	35		
		Iodo-Beads®	154.8		1 bead	92	72	14	14		
		Iodo-Gen®	154.8		1 tube	92	0	10	90		
		Lactoperoxidase	154.8		4.8×10^{-3} µL H_2O_2	102	21	45	34		
Val-Tyr-Val	101.5	Chloramine-T	154.8	7.4	200 nmol	100	4	14	82	Not applicable	
			77.4		100 nmol	100	37	39	24		
		Iodo-Beads®	154.8		1 bead	86	82	11	7		
		Iodo-Gen®	154.8		1 tube	99	23	16	61		
		Lactoperoxidase	154.8		4.8×10^{-3} µL H_2O_2	100	30	38	32		
Gly-Gly-Tyr-Arg	100.5	Chloramine-T	154.8	7.4	200 nmol	100	0	0	100		
			77.4		100 nmol	100	28	43	30		
		Iodo-Beads®	154.8		1 bead	74	88	6	6		
		Iodo-Gen®	154.8		1 tube	98	9	4	87		
		Lactoperoxidase	154.8		4.8×10^{-3} µL H_2O_2	102	28	44	28		
Leucine enkephalin	99.0	Chloramine-T	154.8	7.4	200 nmol	100	12	29	59		
			77.4		100 nmol	100	44	36	20		
		Iodo-Beads®	154.8		1 bead	94	72	16	12		
		Iodo-Gen®	154.8		1 tube	94	15	25	60		
		Lactoperoxidase	154.8		4.8×10^{-3} µL H_2O_2	87	37	41	22		
Neurotensin	10.14	Chloramine-T	7.53	7.4	10 nmol	100	59	32	9	0	0
			15.05		20 nmol	91	30	55	10	5	0
			22.58		30 nmol	100	5	27	14	18	36
			30.10		40 nmol	109	0	4	4	21	71
		Iodo-Beads®	15.05		1 bead	82	83	17	0	0	0
		Iodo-Gen®	15.05		1 tube	41	22	78	0	0	0
		Lactoperoxidase	15.05		4.8×10^{-4} µL H_2O_2	91	35	50	15	0	0
Obestatin	8.83	Chloramine-T	15.05	8.2	20 nmol	119	0	0	28	60	12
			45.15		60 nmol	119	0	0	0	0	100
			15.05		20 nmol	86	6	17	78	0	0
			7.53	7.4	10 nmol	100	43	38	19	0	0
		Iodo-Beads®	15.05		1 bead	62	100	0	0	0	0
		Iodo-Gen®	15.05		1 tube	71	13	13	60	0	13
		Lactoperoxidase	15.05		4.8×10^{-4} µL H_2O_2	81	24	41	35	0	0

[a] Peptide recovery estimated based on sum of corrected peak areas in comparison with non-iodinated reference peptide.
[b] Area% values for Leu-Tyr, Leu-Leu-Tyr, Val-Tyr-Val, Gly-Gly-Tyr-Arg and leucine enkephalin are corrected for estimated relative response factors (*i.e.* area response vs. non-iodinated peptide): mono-iodinated peptide: 2.81, 2.39, 2.43, 2.12 and 1.74, respectively; di-iodinated peptide: 3.46, 3.50, 3.34, 3.23 and 2.52, respectively.

shifts of the aromatic alpha band could be calculated: +8.3 nm for non-iodinated to mono-iodinated peptide and +3.6 nm for mono-iodinated to di-iodinated peptide. These findings correspond to bathochromic shifts mentioned in literature [19–21].

A bathochromic and hyperchromic shift of the p band of tyrosine upon iodination [21] is confirmed by the relative response factors obtained on the single-tyrosine containing iodinated peptides for UV detection at 215 nm (Table 1). An overlay of the UV spectra obtained on leucine enkephalin and its mono- and di-iodinated derivatives is presented in Fig. 3.

3.3. Chromatography

Two different RP-18 columns (Alltima HP AQ and Vydac P&P: pore size 100 and 300 Å, respectively) were investigated for their suitability for the analysis of the model peptides. It was found that the 100 Å column is suitable for the analysis of iodinated LY, VYV, LLY, GGYR and leucine enkephalin, although not for neurotensin and obestatin. The larger peptides (*i.e.* leucine enkephalin, neurotensin and obestatin) and their iodinated species can be analyzed using the second column (*i.e.* Vydac P&P 300 Å). In all cases, non-iodinated peptides,

Table 2
Spectral data on iodinated model peptides

Model peptide	Most abundant peaks in mass spectrum (*m/z*)				
	NIP	MIP	DIP	3IP	4IP
Leu-Tyr	295.0 $[M+H]^+$	420.9 $[M+H]^+$	546.8 $[M+H]^+$		
Leu-Leu-Tyr	408.0 $[M+H]^+$	533.9 $[M+H]^+$	659.8 $[M+H]^+$		
Val-Tyr-Val	380.0 $[M+H]^+$	505.9 $[M+H]^+$	631.7 $[M+H]^+$		
Gly-Gly-Tyr-Arg	452.2 $[M+H]^+$ 226.7 $[M+2H]^{2+}$	578.1 $[M+H]^+$ 289.6 $[M+2H]^{2+}$	704.0 $[M+H]^+$ 352.5 $[M+2H]^{2+}$	Not applicable	
Leucine enkephalin	556.1 $[M+H]^+$	681.9 $[M+H]^+$	807.8 $[M+H]^+$		
Neurotensin	837.6 $[M+2H]^{2+}$ 558.4 $[M+3H]^{3+}$	899.9 $[M+2H]^{2+}$ 600.5 $[M+3H]^{3+}$	962.9 $[M+2H]^{2+}$ 642.5 $[M+3H]^{3+}$	1026.1 $[M+2H]^{2+}$ 684.5 $[M+3H]^{3+}$	1088.9 $[M+2H]^{2+}$ 726.4 $[M+3H]^{3+}$
Obestatin	1258.9 $[M+2H]^{2+}$ 839.9 $[M+3H]^{3+}$ 630.4 $[M+4H]^{4+}$	1321.8 $[M+2H]^{2+}$ 881.6 $[M+3H]^{3+}$ 661.5 $[M+4H]^{4+}$	1384.8 $[M+2H]^{2+}$ 924.1 $[M+3H]^{3+}$ 693.4 $[M+4H]^{4+}$	1447.7 $[M+2H]^{2+}$ 965.7 $[M+3H]^{3+}$ 724.5 $[M+4H]^{4+}$	1510.5 $[M+2H]^{2+}$ 1007.4 $[M+3H]^{3+}$ 755.6 $[M+4H]^{4+}$

Model peptide	Absorbance maximum of aromatic α band of tyrosine residue (nm)[a]		
	NIP	MIP	DIP
Leu-Tyr	275.0 [0.5] ($n=5$)	282.8 [1.1] ($n=5$)	286.8 [0.6] ($n=5$)
Leu-Leu-Tyr	275.8 [0.7] ($n=4$)	283.5 [0.0] ($n=4$)	287.1 [0.0] ($n=5$)
Val-Tyr-Val	275.0 [1.0] ($n=5$)	283.0 [0.7] ($n=5$)	286.5 [1.2] ($n=5$)
Gly-Gly-Tyr-Arg	273.6 [1.0] ($n=5$)	282.9 [0.9] ($n=2$)	285.9 [0.0] ($n=3$)
Leucine enkephalin	275.2 [0.8] ($n=6$)	283.7 [1.0] ($n=5$)	287.7 [0.7] ($n=4$)

[a] Standard deviations are given between square brackets.

Table 3
LC data on iodinated model peptides

Model peptide	Column	Linear gradient (0 → 60 min)	Retention time (min)[a]				
			NIP	MIP	DIP	3IP	4IP
Leu-Tyr	1	8 → 30% ACN	13.07 [0.08] ($n=5$)	33.05 [0.13] ($n=5$)	49.58 [0.25] ($n=5$)		
Leu-Leu-Tyr	1	10 → 50% ACN	20.58 [0.08] ($n=4$)	32.40 [0.07] ($n=4$)	41.20 [0.14] ($n=5$)		
Val-Tyr-Val	1	10 → 50% ACN	13.34 [0.05] ($n=5$)	22.50 [0.08] ($n=5$)	28.96 [0.11] ($n=5$)	Not applicable	
Gly-Gly-Tyr-Arg	1	5 → 30% ACN	6.54 [0.11] ($n=5$)	16.45 [0.09] ($n=2$)	26.76 [0.37] ($n=4$)		
Leucine enkephalin	1	10 → 70% ACN	21.74 [0.08] ($n=5$)	26.87 [0.04] ($n=5$)	30.61 [0.07] ($n=5$)		
	2	10 → 70% ACN	14.95 [0.06] ($n=6$)	19.43 [0.05] ($n=5$)	22.02 [0.07] ($n=5$)		
Neurotensin	2	10 → 70% ACN	14.58 [0.07] ($n=7$)	17.74 [0.10] ($n=7$)	20.57 [0.11] ($n=5$)	22.68 [0.09] ($n=3$)	24.42 [0.13] ($n=2$)
Obestatin	2	10 → 95% ACN	14.25 [0.18] ($n=6$)	15.21 [0.12] ($n=4$)	15.97 [0.10] ($n=5$)	16.70 [0.03] ($n=2$)	18.56 [0.09] ($n=3$)

Column 1 = Alltima HP AQ C_{18}, 250 × 4.6 mm, 5 μm; column 2 = Vydac P&P C_{18}, 250 × 4.6 mm, 5 μm.
[a] Standard deviations are given between square brackets.

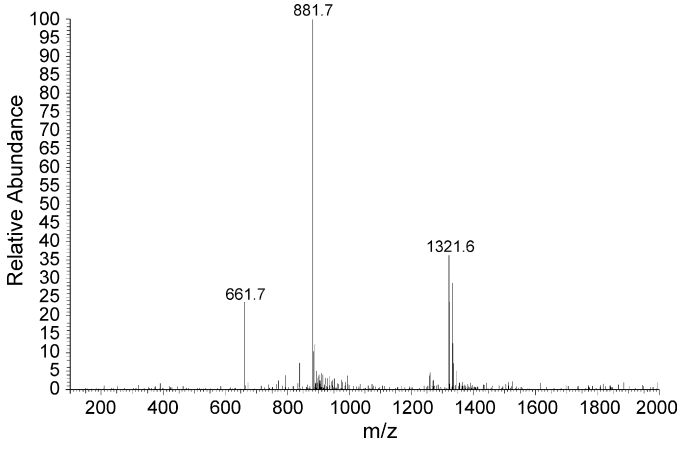

Fig. 1. Typical mass spectrum obtained on mono-iodinated obestatin.

as well as mono- and di-iodinated peptides (and for neurotensin and obestatin, even tri- and tetra-iodinated peptides) could be resolved using standard HPLC methods with acetonitrile, water and 0.1% (m/v) formic acid as mobile phase constituents (Table 3). A typical total ion current (TIC) chromatogram obtained on iodinated leucine enkephalin is depicted in Fig. 4.

Due to the increasing lipophilicity as the iodination degree increases, an increase in retention time is expected. However, up till now, no correlation has been published. Using our seven model peptides (with leucine enkephalin analyzed on both 100 and 300 Å column, so $n=8$), a relationship could be observed between the ratio of the percentage acetonitrile at the retention time of the MIP versus NIP and DIP versus MIP species (corrected for hold-up time) and the molecular mass of the iodi-

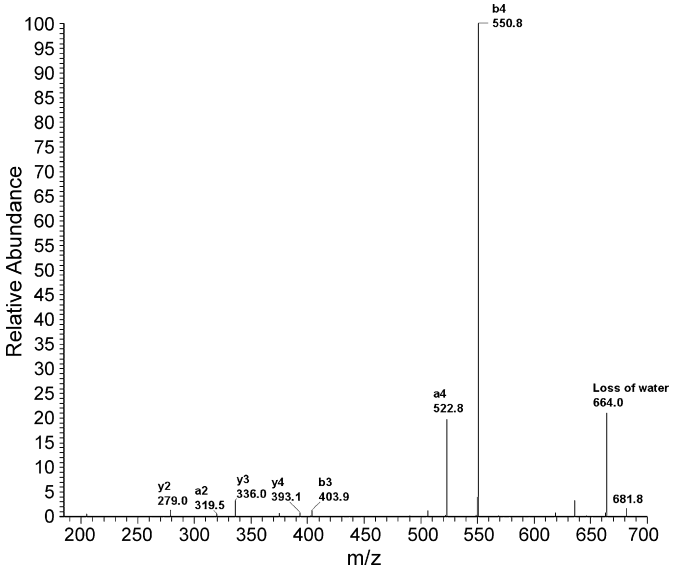

Fig. 2. Typical tandem mass spectrum obtained on mono-iodinated leucine enkephalin (Iodo-Gen® synthesis).

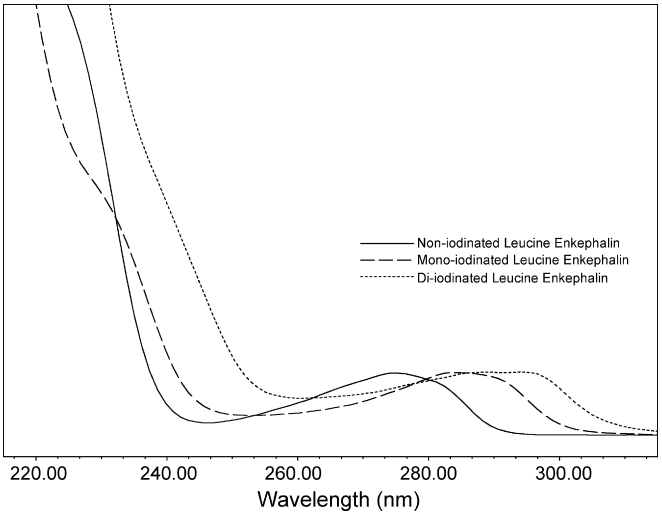

Fig. 3. Overlay UV spectrum obtained on leucine enkephalin and its mono- and di-iodinated derivatives (*y*-axis scaled for better view of tyrosine alpha band shift).

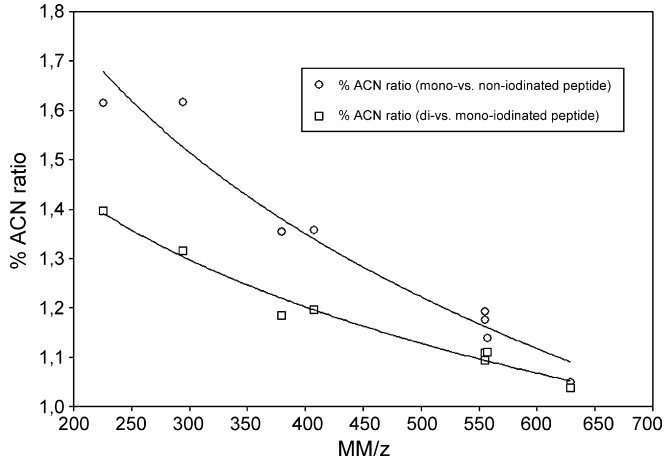

Fig. 5. Relationship between ratio of percentage acetonitrile at retention time of iodinated compounds corrected for hold-up times and MM/*z*.

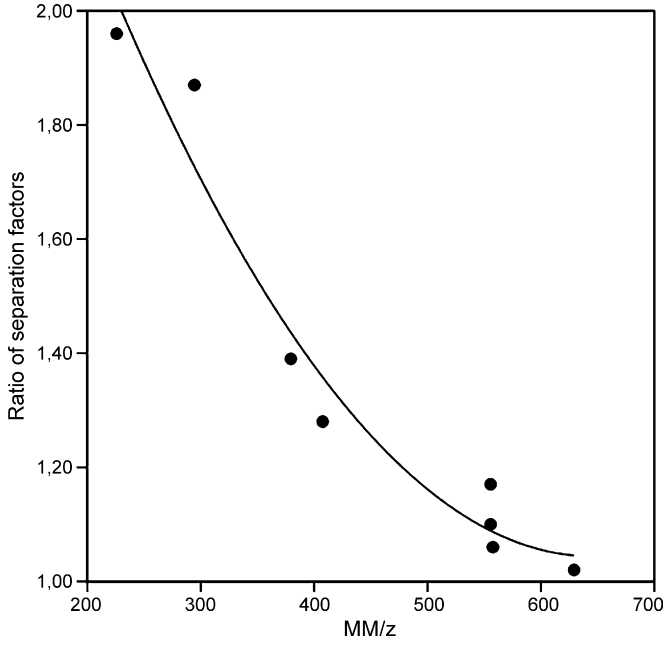

Fig. 6. Relationship between ratio of separation factors ($\alpha_{NIP,MIP}/\alpha_{MIP,DIP}$) and MM/*z*.

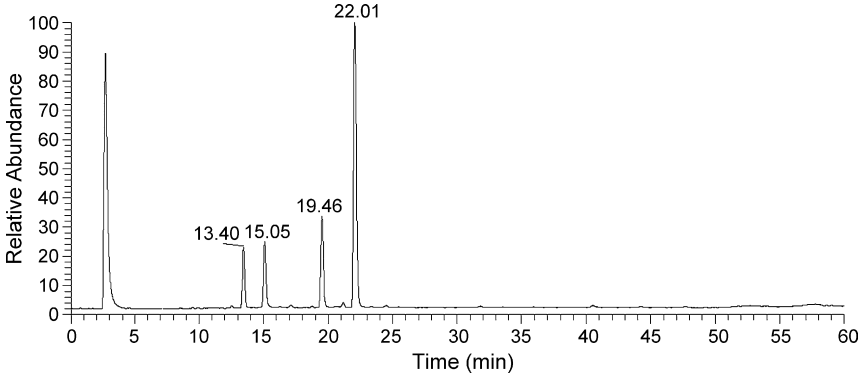

Fig. 4. Typical chromatogram (MS-TIC) obtained on leucine enkephalin iodinated with Iodo-Gen®, using a Vydac P&P 300 Å C_{18} 5 μm (250 × 4.6 mm i.d.) column. The chromatographic conditions were as described in Section 2.9. The gradient program was set as follows: 0–60 min, 10–70% of B (linear gradient). Injection volume: 10 μL. Peaks at 13.4, 15.1, 19.5 and 22.0 min correspond to Iodo-Gen® reagent, non- (NIP), mono- (MIP) and di-iodinated (DIP) peptide, respectively.

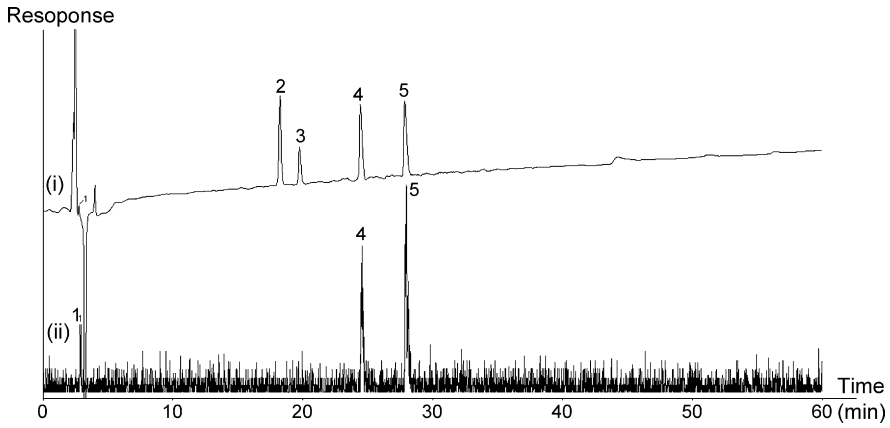

Fig. 7. Typical radio-HPLC chromatograms obtained on ^{125}I labelled leucine enkephalin using an Alltima HP AQ 100 Å C_{18} 5 μm (250 × 4.6 mm i.d.) column: (i) UV at 215 nm and (ii) radioactivity. The chromatographic conditions were as described in Section 2.9. The gradient program was set as follows: 0–60 min, 10–70% of B (linear gradient). Injection volume: 20 μL. Peaks at 2.8, 18.3, 19.8, 24.4 and 27.9 min correspond to (1) free iodine, (2) chloramine-T reagent degradation product, (3) non- (NIP), (4) mono- (MIP) and (5) di-iodinated (DIP) peptide, respectively.

nated peptide divided by its number of charges in acidic medium (*i.e.* pH of mobile phase is 2.7). Empirically, this relationship is mathematically expressed as:

$$\text{for MIP/NIP}: \ \%\text{ACN ratio} = 4.79 - 0.57 \times \ln(\text{MM}/z)$$

$$\text{for DIP/MIP}: \ \%\text{ACN ratio} = 3.19 - 0.33 \times \ln(\text{MM}/z).$$

The *R*-squared values calculated for both models are 0.948 and 0.979, respectively. Higher MM/z values resulted thus in lower acetonitrile percentage ratios (Fig. 5). It was also observed that for peptides with a MM/z of approximately 500 or more, the ratio of the separation factors between the respective non- and mono-iodinated peptide pair ($\alpha_{\text{NIP,MIP}} = t_{R'}[\text{MIP}]/t_{R'}[\text{NIP}]$) and mono- and di-iodinated peptide pair ($\alpha_{\text{MIP,DIP}} = t_{R'}[\text{DIP}]/t_{R'}[\text{MIP}]$) decreases to 1, whereas for the dipeptide Leu-Tyr the ratio was found to be equal to 2 (Fig. 6). As a consequence, peptides with higher MM/z values are expected to present equal separation between mono- and di-iodinated species as to non- and mono-iodinated species.

Although several models exist for the prediction of retention times of peptides from digested proteins in reversed-phase HPLC [22], no model on the retention times of iodinated peptides has been proposed thus far. The current findings can be of practical use for the prediction of the retention times of new iodinated peptide species.

3.4. Radio-HPLC analysis

Radioiodination of leucine enkephalin with carrier-added ^{125}I using the chloramine-T technique resulted in two radiolabelled compounds: mono- and di-iodinated peptide (Fig. 7). The peak heights obtained with the radioactivity detector confirm the area response factors calculated for UV detection at 215 nm (Table 1).

As demonstrated by this case, where two iodinated species were formed and the native peptide is still present, quality control on radioiodinated peptides using a suitable separation technique (such as liquid chromatography as described here) is recommended for radiotracer and imaging studies to determine the chemical and radiochemical purity.

4. Conclusions

Iodinated peptides were separated by HPLC using standard 100 Å and/or 300 Å C_{18} columns with acetonitrile–water mobile phase gradients and 0.1% (m/v) formic acid as ion pairing agent. The iodinated peptides were identified by ultraviolet spectrophotometry and mass spectrometry. A relationship has been found between the separation of non-, mono- and di-iodinated peptides and the mass-to-charge ratio (MM/z) of the native peptide.

This analytical methodology was applied in the comparison of different synthesis routes. It is concluded that under the conditions applied in these iodination experiments, the Iodo-Beads® technique is inferior in terms of peptide recovery, and formation of mono- and di-iodinated peptides. For iodination of peptides that are not sensitive to the oxidizing agent, the chloramine-T technique is recommended based on its efficiency, simplicity and cost.

The applicability of the methodology was demonstrated on ^{125}I labelled leucine enkephalin. It is recommended to determine the purity of radioiodinated peptides by a suitable separation technique such as radio-HPLC.

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