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ORIGINAL ARTICLE

Metabolic stability of long-acting luteinizing hormone-releasing hormone antagonists

Jin-Feng Yao · Ning Zhou · Yu-Jian Lv · Ruifeng Zhang · Ke-Liang Liu · Ming Xue

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Abstract Long-acting luteinizing hormone-releasing hormone (LHRH) antagonists designed to be protease resistant consisted of a series of novel decapeptides structurally similar to LHRH. The aim of this study was to evaluate the in vitro metabolic stability of the LHRH decapeptides using pancreatin and homogenates models and identify the metabolites in rat liver homogenate for the purpose of illustrating the metabolic features of the decapeptides. The major metabolites in rat liver homogenate were identified by LC–ESI-MS^n. The half-lives of the 11 LHRH decapeptides were from 44 to 330 min in the pancreatin model. The half-lives of the five decapeptides in rat liver, kidney and lung homogenates were between 8 and 462 min. The most stable decapeptides were the LY616 and LY608 peptides with half-lives of 36 min in liver homogenate. Two major cleavage sites were found by analysing the metabolites of the LY618 peptide in rat liver homogenate, between the Pal^3-Ser^4 and the Leu^7-$Ilys^8$ peptide bonds. The major metabolites were produced via cleavages of peptide bonds at these sites, and further metabolic reactions such as hydroxylation, oxidative dechlorination, alcohol dehydration and isopropyl dealkylation were also observed.

J.-F. Yao · M. Xue (✉)
Department of Pharmacology, School of Basic Medical Sciences, Capital Medical University, No. 10 Xitoutiao, You An Men, Beijing 100069, China
e-mail: xuem@ccmu.edu.cn

N. Zhou · Y.-J. Lv · K.-L. Liu
Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China

R. Zhang
Department of Anatomy, Yanjing Medical College, Capital Medical University, No. 4 Dadong Rd, Beijing 101300, China

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Introduction

There is a currently renewed interest in the pharmaceutical industry for developing peptides as therapeutic compounds (Wilson et al. 2010) and includes synthetic peptides as analogues of endogenous peptides or derived from natural bio-active peptides (Van den Broek et al. 2008). Recombinant-derived proteins and peptides are becoming increasingly important as therapeutic agents (Modi 1994), however, parenterally administered proteins and peptides tend to be rapidly cleared from circulation by the reticuloendothelial system (RES) or metabolized by serum and liver peptidases leading to loss of biological activity (Kompella and Dani 1996; Ryan et al. 2011). There is usually no activity from oral administration of peptides (Fasano 1998; Mahato et al. 2003; Tang et al. 2004). To improve the therapeutic potential of peptide drugs, it is important to determine their route of metabolism and establish quantitative structure–stability relationships that can be used to improve the metabolic stability of synthetic peptides (Vergote et al. 2008).

Luteinizing hormone releasing hormone (LHRH) is a peptide produced by the hypothalamus and consists of 10 amino acids. LHRH helps with the production of luteinizing hormone (LH) and follicle stimulation hormone (FSH). LH and FSH stimulate the growth of the human body, human reproduction system and physiological processes that may be involved (Herbst 2003). Studies have shown that they also have a positive effect on cancers such as endometrial carcinoma (Stricker 2001), prostatic carcinoma

(Weckermann and Harzmann 2004) and child precocious puberty (Griesinger et al. 2005). Therefore, LHRH analogues have been designed for use in cancer treatments. They include the LHRH agonists such as leuprolide, triptorelin and goserelin, and the LHRH antagonists such as cetrorelix, abarelix and ganirelix. Compared with the LHRH agonists, the LHRH antagonists are more effective at treating cancer, because they can rapidly reduce the secretion of sex hormones due to the effectiveness in blocking LHRH receptors and inhibiting the release of LH and FSH by the hypophysis. Recent studies have revealed some metabolic mechanism of LHRH analogues (Kompella and Dani 1996, 1997; Koushik et al. 2003; Cleverly and Wu 2010). LHRH peptides can be easily hydrolyzed by proteases to become inactive substances (Woodley 1994), resulting in a short half-life in vivo. The modifications of the structures should improve the enzymatic stability of LHRH analogues (Pappa et al. 2011; Manea et al. 2011). In our research, the novel long-acting LHRH antagonists were synthesized to be protease resistant and their bioactivities were evaluated by the testosterone test model. The LHRH antagonists showed longer duration for inhibiting testosterone secretion (Zhou et al. 2007, 2008; Gao et al. 2009, 2010). The 11 antagonists had two general sequences which consisted of 10 amino acids, and the structures of antagonists are shown in Table 1. In this study, we characterized the in vitro metabolic stability of the LHRH peptides in pancreatin and homogenates of rat liver, kidney and lung tissues. The residuals of LHRH antagonists in three tissue homogenates were monitored as a function of time by LC–MS method, and the major degradation products were identified by LC–ESI-MS/MS. These results provide a basis for the design of novel LHRH peptide analogues that have improved metabolic stability.

Methods

Materials

LHRH peptides were synthesized by Beijing Institute of Pharmacology and Toxicology, China, purified by HPLC (purity >99%), and stored at −70°C. Pancreatin and degarelix were purchased from Sigma Ltd. Co. (St. Louis, MO, USA). Acetonitrile and formic acid (99%, HPLC grade) were obtained from Fisher Scientific Products (Fair Lawn, NJ, USA). HPLC grade water was doubly purified with a Milli-Q system (Millipore, Molsheim, France). All other reagents were of the highest grade commercially available.

Instrument and chromatographic-mass conditions

The analyses of LHRH peptides (MW, 1,484–1,720 Da) in pancreatin and homogenates were performed on an Agilent HPLC (Series 1100, Agilent Technology, Palo Alto, CA, USA) and LC–ESI-MS system. The LC–ESI-MS system consisted of a HPLC system (Series 1100) coupled to a Finngan LCQ Deca XP ion-trap spectrometer equipped with an electrospray source (Thermo Finnigan, San Jose CA, USA). The separation column was Hypersil Gold (100 mm × 2.1 mm, i.d. 3 μM). All chromatography was performed at 25°C. The LC–MS system was controlled by Xcalibur® (version 1.3) software. The mobile phase was composed of acetonitrile: water (containing 0.5% v/v formic acid, pH 2.7) = 30:70 (v/v) at a flow rate of 0.2 mL/min. Electrospray MS was performed in positive-ion selected ion monitoring (SIM) mode. MS parameters were optimized for achieving good sensitivity for all decapeptides in one single analytical run. ESI was operated at the capillary temperature of 350°C. The operating conditions were optimized by direct infusion of a mixture of all analytes. Nitrogen was used as sheath gas and aux/sweep gas in the ion trap, and the flow rate was 50 psi. The injection volume for LC experiments was 10 μL.

The mobile phase of HPLC was a mixture of solvent A (acetonitrile) and B (water containing 0.5% (v/v) formic acid) employing gradient elution (from 5:95 to 30:70, v/v, pH 2.7) at a flow rate of 0.2 mL/min. The gradient program was as follows: initial 0–18 min, linear change from A–B (5:95) to A–B (28:72); 18–40 min, linear change from A–B (28:72) to A–B (30:70). The detection wavelength was set at 226 nm. The sample injection volume was 10 μL.

Degradation of LHRH antagonists in pancreatin

For cassette dosing, 11 LHRH antagonists were equally divided into three groups according to their polarity. Group A contained LY603, LY609 and TRD122, group B consisted of LY602, LY605, LY607 and LY616, and group C included LY608, LY617, LY618 and LY663. Prior to testing, the pancreatin solution (pH 7.4, 100 μg/mL) was preheated for 15 min at 37°C. The reaction was initiated by the addition of a mixed solution of group A at a final concentration of 100 μg/mL, and then the reaction was incubated at 37°C for 4 h. Samples (100 μL) were withdrawn at different time intervals (0, 1, 3, 5, 10, 15, 30, 45, 60, 90, 120 and 240 min), immediately mixed with 100 μL acetonitrile to terminate enzymatic activity and vortexed. The mixtures were centrifuged at 10,000*g* for 10 min at 4°C, the resulting supernatants (10 μL) were withdrawn and examined using HPLC. An appropriate blank control (i.e. no pancreatin) and positive control (degarelix) were prepared in a similar manner. The half-life values of LHRH peptides in the pancreatin system were calculated to quantitate the metabolic stability. The stabilities of group B and C were determined using the same method.

Table 1 Structures of long-acting LHRH antagonists derived from sequence a and b

Sample	X	Aaa^3	Yy^5	Zz^6	Aaa^8	MW
Sequence a: X-D-Nal^1-D-Cpa^2-Aaa^3-Ser^4-Yy^5-Zz^6-Leu^7-Aaa^8-Pro^9-D-Ala^{10}-NH_2						
LY602	Cbm	D-Phe^3	Mop^5	D-Aph (Ac)^6	$ILys^8$	1,575.3
LY603	Cbm	D-Pal^3	Mop^5	D-Aph (Ac)^6	$ILys^8$	1,576.3
LY608	Cbm	D-Pal^3	Aph (Hor)^5	D-Aph (DHE-Cbm)^6	$ILys^8$	1,720.4
LY663	Cbm	D-Phe^3	Mop^5	D-Aph (Ac)^6	Arg^8	1,561.2
TRD122	Ac	D-Phe^3	Gln^5	D-Mop^6	Arg^8	1,484.1

Sample	X	Aaa^5	Aph^6 (4–R)	MW
Sequence b: X-D-Nal^1-D-Cpa^2-D-Pal^3-Ser^4-Aaa^5-D-Aph^6-Leu^7-$Ilys^8$-Pro^9-D-Ala^{10}-NH_2				
LY605	Ac	Aph (Hor)^5	Nic^6	1,694.3
LY607	Ac	Aph (Bio)^5	Cbm^6	1,718.5
LY609	Ac	Aph (Nic)^5	Cbm^6	1,597.3
LY616	Ac	Aph (Hor)^5	Mor-Cbm^6	1,702.3
LY617	Ac	Mup^5	Cbm^6	1,605.3
LY618	Cbm	Aph (Hor)^5	Cbm^6	1,633.2

Ilys N′-isopropyl-lysine, *Nal* 3-(2-naphthyl)-alanine, *Cpa* 4-chlorophenylalanine, *Pal* 3-(3-pyridyl)-alanine, *Mop* 4-morpholinemethylphenylalanine, *Aph* 4-aminophenylalanine, *Mup* 4-morpholineureaphenylalanine, *Nic* nicotinicacid, *Bio* biotin, *Hor*  , *Cbm*  , *DHE-Cbm*  , *Mor-Cbm* 

Degradation of LHRH antagonists in tissue homogenates

Three male Sprague–Dawley rats (250 ± 20 g) were obtained from the Animal Center of Capital Medical University (ACCMU, Beijing, China). Before the experiment, the rats were fasted overnight with free access to water and were killed by cervical dislocation. The liver, lungs and kidneys were quickly removed from each rat, washed in ice-cold 0.9% sodium chloride solution, weighed and cut into small pieces, diluted with 4 mL of ice-cold Tris–HCl buffer (50 mM, pH 7.4) per 1 g of tissue. For each tissue, the samples from the three rats were pooled and homogenized using a Teflon digital homogenizer. The homogenates were centrifuged (3,000*g*) for 10 min at 4°C. The total protein concentration was determined using the bicinchoninic acid (BCA) colorimetric assay (Pierce Biotechnology, Rockford, IL, USA) with bovine serum albumin as a reference standard. The homogenates were diluted to 2.5 mg protein/mL with Tris–HCl buffer as necessary.

According to the stabilities of the 11 decapeptides in pancreatin, LY616, LY608 and LY617 were the most stable compounds, and LY618 and LY609 were the most unstable peptides. To seek metabolism characteristic of the peptides, the five compounds mentioned above were further studied in the homogenates. Prior to the study, the homogenate (pH 7.4, 2.5 mg protein/mL) was preheated for 15 min at 37°C. A mixture of five decapeptides was added to the homogenates at a final concentration of 100 μg/mL, and then the reaction was initiated by heating at 37°C in a water bath. The samples (100 μL) were withdrawn at different time intervals (0, 1, 3, 5, 10, 15, 30, 45, 60, 90, 120 and 240 min), mixed with 100 μL acetonitrile to terminate the activity and vortexed immediately. The mixtures were centrifuged at 10,000*g* for 10 min at 4°C, the resulting supernatants (10 μL) were withdrawn and measured using LC–ESI-MS. A ThermoFinnigan LCQ Deca XP mass spectrometer (ThermoFinnigan, CA, USA) was used to analyse the degradation products of the decapeptides. The half-life values of LHRH antagonists in the tissue homogenates system quantitated their metabolic stability.

Results

Stability in pancreatin

LHRH antagonists were qualitatively measured by retention time and were quantified by an external standard method using the peak area of the sample. The formula of $t_{1/2} = 0.693/k$ was used to calculate the half-life values. The calibrations, LnC-t equations and low limits of quantification (LOQ) of the method are listed in Table 2. The results showed that the calibration curves of all the peptides were linear in the concentration range of 1.0–400 μg/mL; the correlation coefficients were all more than 0.9991.

The LOQ of 13 peptides were within 1.0–10 μg/mL. The half-life of degarelix was 84 min and the half-life values of 11 LHRH peptide analogues were from 44 to 330 min. Among the 11 decapeptides, the half-life value of LY616 was the longest. These results indicated that the pancreatin model was suitable to predict the metabolic stability of the decapeptides in vitro, and the stability of the eight decapeptides was better than that of degarelix.

Stability in rat tissue homogenates

The metabolic stabilities of five LHRH peptides in tissue homogenates are shown in Fig. 1. Five peptides were monitored over 240 min in rat liver, kidney and lung homogenates. After 1 h incubation, the residuals of the initial five peptides were less than 30% in liver homogenate. In contrast, more than 60% of the five peptides except for LY618 still remained in the kidney and lung homogenates. No clear degradation was observed in control experiments over 240 min of incubation. The rank order of the degradation rate of the five peptides in the three homogenates was liver > kidney > lung. The half-life values of the five peptides in the three matrices were calculated (Table 3) and the half-lives were between 8 and 462 min. The half-life of LY618 in liver homogenate was the shortest at 7.5 min, whereas the half-lives of LY616 and LY608 were the longest at 36 min. The half-lives of the five peptides in the kidney and lung homogenates were longer than in liver homogenate, indicating the five peptides were relatively stable in kidney and lung tissues.

The results showed that the stabilities of LY616 and LY608 were better than that of the other peptides. Among the tested peptides, LY618 was the most unstable one, especially when it was incubated in liver homogenate. Therefore, to determine the enzyme cleavage site, the LY618 peptide was incubated in rat liver homogenate for 30 min, and then the fragments and metabolites were identified with LC–ESI-MS^n. The blank liver homogenate and standard solution were used for the blank and positive control, respectively. The structure and the suggested proteolytic cleavage sites of LY618 in rat liver homogenate are illustrated in Fig. 2. Two cleavage sites were identified; one was at the peptide bond between Pal and Ser, and the other at the Leu and Ilys sites. Four metabolites including M1, M2, M3 and M4 were formed and identified. The proposed metabolic and fragmentation pathway of LY618 is shown in Fig. 4.

Metabolite M1 was observed as a protonated molecule $[M + H]^+$ at *m/z* 390, with a retention time of 42.25 min (Fig. 3a). M1 was formed by the following metabolic reactions: first, LY618 was hydrolyzed between the Leu and Ilys peptide bond by a protease to produce a tripeptide (356 Da) and a heptapeptide (1,295 Da) (Fig. 2). Then the tripeptide, which was composed of Ilys, Pro and D-Ala-NH_2, was increased by 34 Da via the following two reactions on alanine (Fig. 4a): the first occurred at the secondary amine site to form hydroxylamin followed by demethylation and dihydroxylation. The metabolite M1 was formed via the above reactions from the tripeptide. A series of characteristic fragment ions at *m/z* 328 and 284 for M1 were detected in the MS^2 spectra (Fig. 5a). The ion at *m/z* 328 was formed by loss of a H_2O (18 Da) and the $CONH_2$ group from M1, to produce the ion at *m/z* 284 via the loss of CO_2. The proposed fragmentation pathway of the tripeptide is shown in Fig. 4a.

M2, eluted at 52.11 min (Fig. 3b), gave rise to the protonated molecule $[M + H]^+$ at *m/z* 1,259 that was formed by the following reactions (Fig. 4b): first the heptapeptide (1,295 Da) was generated by the hydrolysis of LY618 between the Leu and Ilys peptide bond (Fig. 2), then the heptapeptide was reduced 36 Da by the oxidative dechlorination reaction of D-*p*-chlorophenyl alanine and the

Table 2 Calibrations, LnC-t equations, LOQ and half-lives of peptides in pancreatin ($n = 3$)

Decapeptides	Calibration of external standard method	LOQ (μg/mL)	LnC-t equations	$t_{1/2}$ (min)
Degarelix	$y = 27{,}832x - 75{,}661$ ($r = 0.9999$)	5	$\mathrm{LnC} = -0.0081t + 5.7934$ ($r = 0.9991$)	84
LY602	$y = 23{,}155x - 138{,}969$ ($r = 0.9998$)	5	$\mathrm{LnC} = -0.0103t + 5.2088$ ($r = 0.9972$)	210
LY603	$y = 21{,}632x - 109{,}818$ ($r = 0.9996$)	5	$\mathrm{LnC} = -0.0433\mathrm{t} + 5.316$ ($r = 0.9954$)	108
LY616	$y = 24{,}181x - 141{,}070$ ($r = 0.9995$)	5	$\mathrm{LnC} = -0.0021t + 5.3733$ ($r = 0.9974$)	330
LY617	$y = 17751x - 107{,}389$ ($r = 0.9996$)	10	$\mathrm{LnC} = -0.0028t + 4.1767$ ($r = 0.9907$)	252
LY605	$y = 24576x - 81{,}296$ ($r = 0.9999$)	5	$\mathrm{LnC} = -0.0054t + 4.7718$ ($r = 0.9914$)	126
LY607	$y = 31669x - 130{,}296$ ($r = 0.9991$)	5	$\mathrm{LnC} = -0.0028t + 4.5579$ ($r = 0.9947$)	246
LY618	$y = 40246x - 325{,}362$ ($r = 0.9998$)	1	$\mathrm{LnC} = -0.0109t + 5.9705$ ($r = 0.9868$)	66
LY663	$y = 24740x - 181{,}899$ ($r = 0.9993$)	5	$\mathrm{LnC} = -0.0036t + 5.0787$ ($r = 0.9900$)	192
LY608	$y = 41225x - 394{,}502$ ($r = 0.9996$)	1	$\mathrm{LnC} = -0.0024t + 4.7408$ ($\mathrm{r} = 0.9983$)	288
LY609	$y = 30512x - 336{,}159$ ($r = 0.9993$)	1	$\mathrm{LnC} = -0.0116t + 4.5435$ ($r = 0.9801$)	60
TRD122	$y = 27053x - 80{,}775$ ($r = 0.9999$)	5	$\mathrm{LnC} = -0.0159t + 6.0842$ ($r = 0.9889$)	44

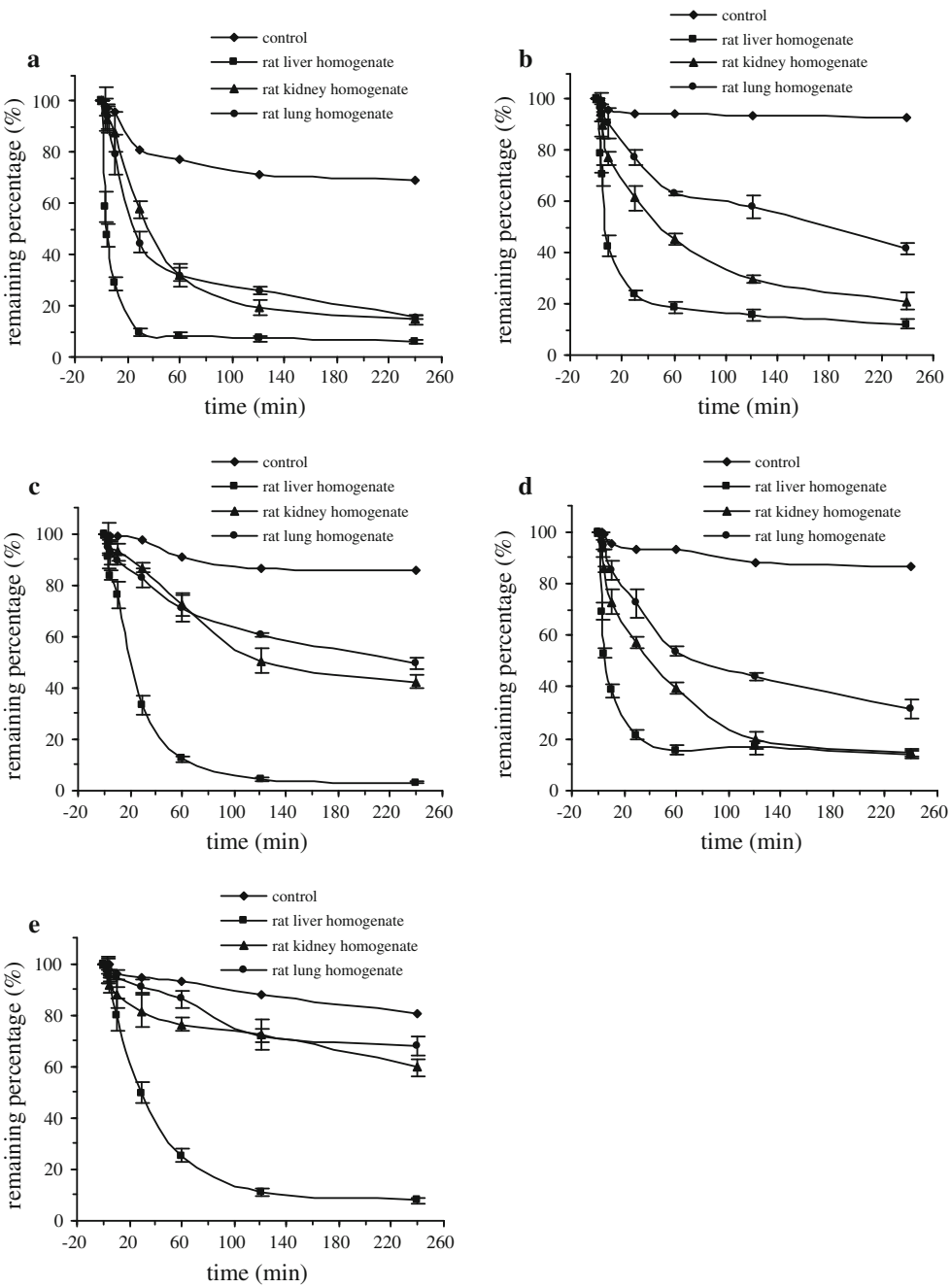

Fig. 1 Metabolic stability of five decapeptides in rat liver, kidney and lung homogenates. The residual rates of five peptides at 0 min were set to 100% and the Tris–HCl solutions without homogenates was used for the blank control, $n = 3$. **a** LY618, **b** LY609, **c** LY617, **d** LY616, **e** LY608

alcohol dehydration reaction of the serine site. The MS^2 spectra at *m/z* 1,011 contained the characteristic ion of M2 (Fig. 5b), which was formed via the loss of three protons and the side chain located in the Aph amino acid (245 Da). The suggested fragmentation pathway is shown in Fig. 4b.

M3 and M4, which were detected at retention times of 54.06 and 55.97 min, respectively (Fig. 3c, d), were generated from the heptapeptide fragment. The heptapeptide containing the amino acid series Ser^4-Aph-$(Hor)^5$-D-Aph^6(4'-Cbm)-Leu^7-$Ilys^8$-Pro^9-D-Ala^{10}-NH_2 (MW: 1,063 Da) was produced by the hydrolysis of LY618 at the Pal^3-Ser^4 peptide bond, then different metabolic reactions of the heptapeptide resulted in different metabolites.

Metabolite M3 ($[M + 2H]^{2+}$: *m/z* 522) was formed by an alcohol dehydration reaction of the hydroxyl group attached to the Ser^4 site of the heptapeptide (Fig. 4c). A series of characteristic fragment ions at *m/z* 504, and 184 of M3 appeared in the MS^2 spectra (Fig. 5c). The fragment *m/z* 504 was generated from M3 via the loss of $-NH_3^+$ attached to D-Aph^6 (4′-Cbm) and a proton, and the ion *m/z* 184, a dipeptide fragment consisting of Pro^9 and D-Ala^{10}-NH_2, could be formed through the cleavage

Table 3 The half-lives of five peptides in rat tissue homogenates at pH 7.4 and 37°C ($n = 3$)

Peptides	Tissue homogenates	Half-life (min)
LY609	Liver	8.0 ± 0.57
	Kidney	52.5 ± 1.63
	Lung	144.4 ± 4.24
LY617	Liver	25.6 ± 2.69
	Kidney	126.0 ± 6.04
	Lung	173.3 ± 11.51
LY618	Liver	7.5 ± 0.65
	Kidney	54.1 ± 3.35
	Lung	69.3 ± 6.70
LY616	Liver	36.5 ± 3.43
	Kidney	364.7 ± 5.31
	Lung	462.0 ± 13.88
LY608	Liver	36.3 ± 2.36
	Kidney	346.5 ± 3.84
	Lung	407.6 ± 9.80

between Ilys[8] and Pro[9]. Therefore, both the metabolite M3 and M4 can produce the fragment *m/z* 184.

Metabolite M4, which gave rise to the double-charged ion at *m/z* 511 ($[M + 2H]^{2+}$) was formed via an isopropyl dealkylation reaction (C_3H_6, 42 Da) of Ilys[8] on the heptapeptide (Fig. 4c). The typical fragment ions were at *m/z* 184 and 492 (Fig. 5d), the ion at *m/z* 492 was produced from M4 via the loss of $-NH_3^+$ attached to D-Aph[6] (4′-Cbm) and two protons. The ion at *m/z* 184 was the same fragment ion of M3 and M4, moreover, it was formed in the same way, thus indicating that both M3 and M4 were from the same heptapeptide fragment.

Discussion

Some proteolytic enzymes occur exclusively in the liver, kidney or other tissues. To identify the influence of these organs on enzymatic degradation, homogenates of liver or kidney can be used for enzymatic stability studies (Powell

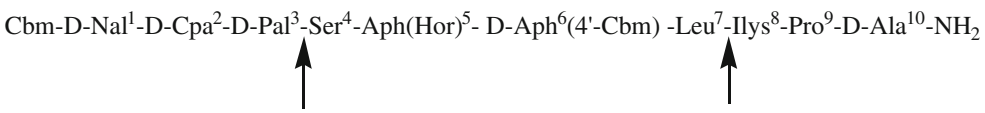

Fig. 2 Proposed metabolic cleavage sites of LH618 in rat liver homogenate. *Arrowheads* indicate the cleavage sites in LY618

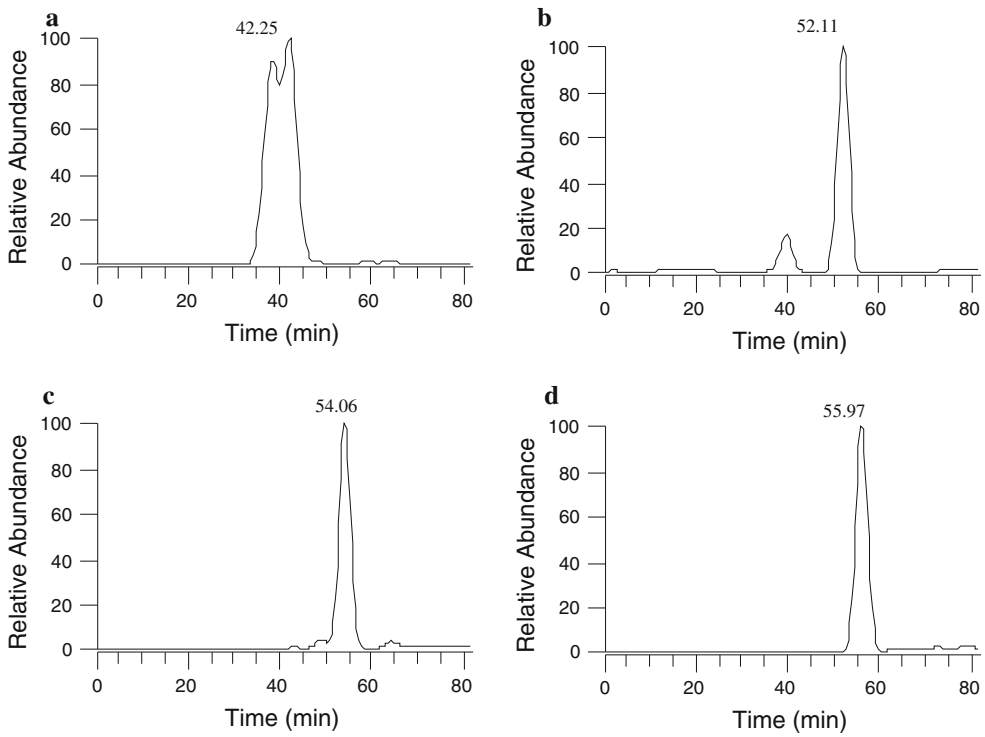

Fig. 3 LC–MS[2] chromatograms of LY618 metabolites in rat liver homogenate

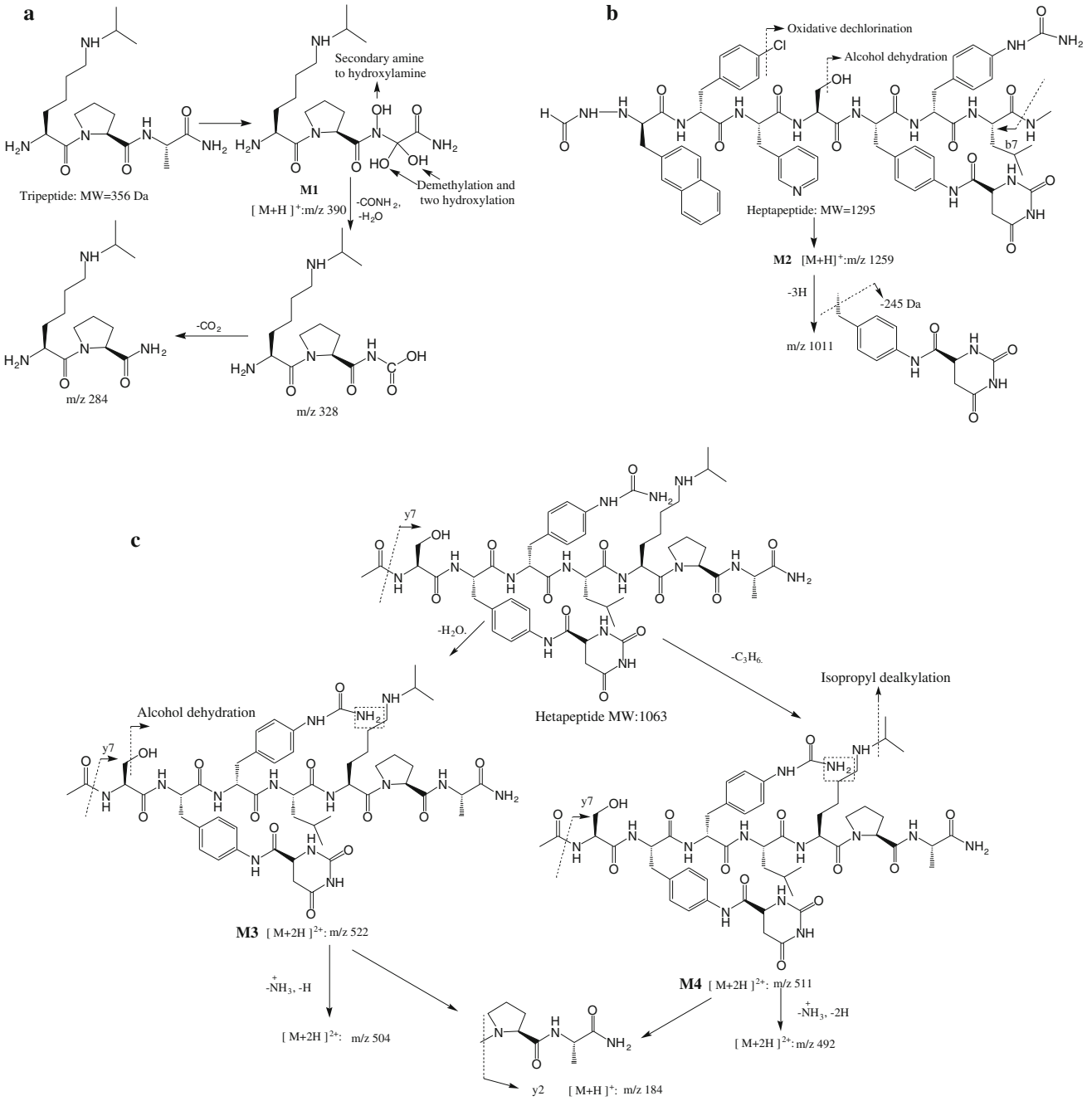

Fig. 4 Proposed major metabolites, fragment and metabolic pathways of LY618 in rat liver homogenate

et al. 1992; Boulanger et al. 1992). The instability of several peptides in the biological matrix has been reported (Darby et al. 2001; Fanciulli et al. 2006, 2007; Berna et al. 2008). Rat liver, kidney and lung homogenates were applied to assess metabolic stability of the LHRH decapeptides in this study. The factors influencing the elimination rate of protein and peptide include the molecular weight and physical and chemical properties of the molecules (Meijer and Ziegler 1993). The metabolic mechanism responsible for peptide breakdown varies depending on the molecular weight. Peptides with molecular weights between 500 and 1,000 Da are generally metabolized by liver, while peptides with molecular weights between 1,000 and 5,000 Da are metabolized by kidney, although the different metabolic mechanisms may overlap with each other (Braeckman 1997). Our study has demonstrated that five decapeptides with molecular weights between 1,597 and 1,720 Da were rapidly metabolized by three tissue

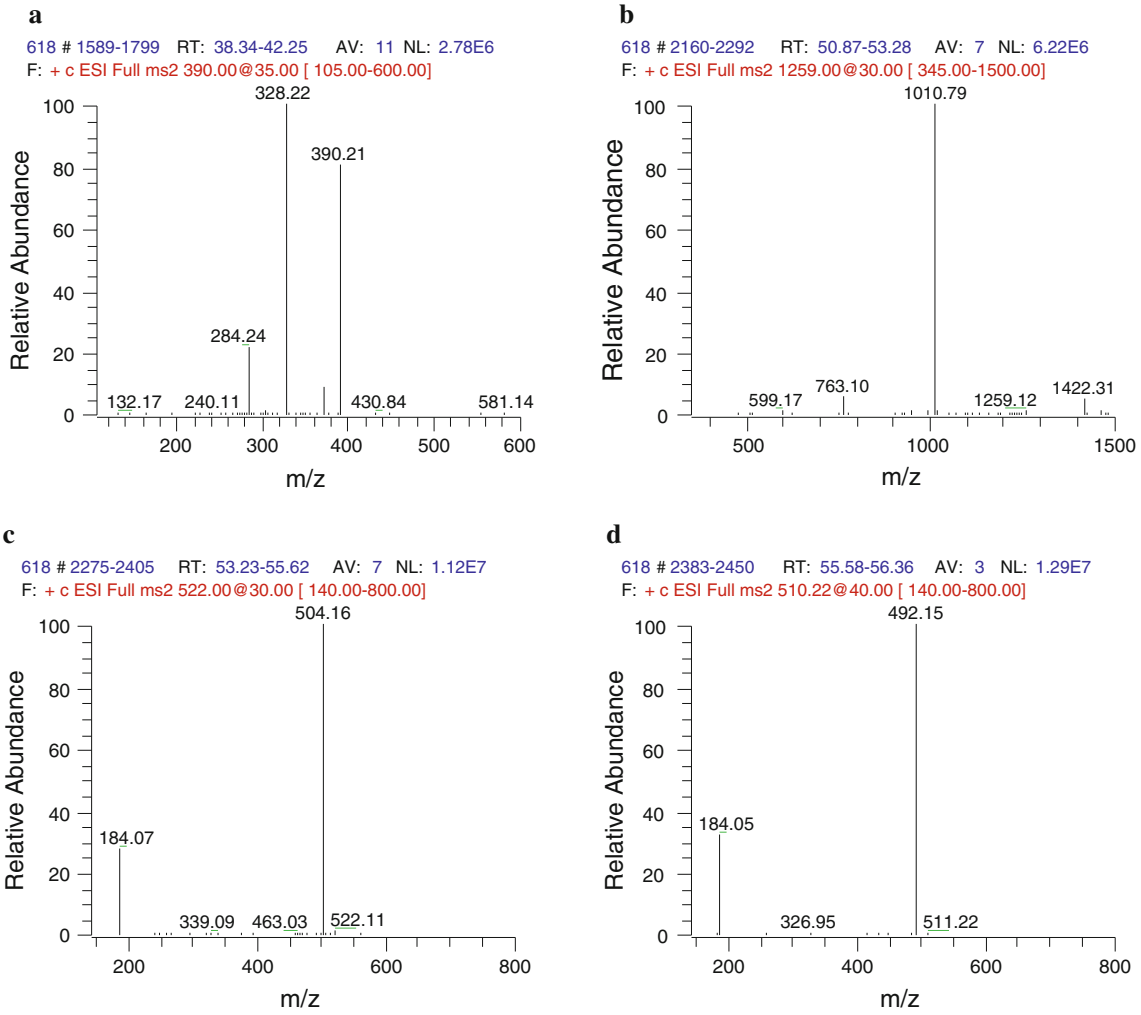

Fig. 5 MS/MS product ion spectra of LY618 metabolites in rat liver homogenate

homogenates, and the half-life values of these peptides in rat homogenates were between 8 and 462 min. The rank order of the degradation rate of the five peptides in three homogenates was liver > kidney > lung. Our result is not fully consistent with Braeckman's (1997), this maybe explained by the overlapping of the different metabolic mechanisms.

The chemical instability of peptides and proteins is defined by the extent of modifications to the amino acid residues, such as oxidation, reduction, hydrolysis or deamidation. The factors influencing both the chemical and physical instability of the amino acids, peptides and proteins have been described and specified by Reubsaet et al. (1998a, b). Recent research suggested that LHRH peptides could be cleaved by metalloendopeptidase EC 3.4.24.15 (EP24.15) at the covalent bond between the fifth and sixth residue of the decapeptide [Tyr(5)-Gly(6)] (Wu et al. 2009). Our research showed that the decapeptide was first cleaved by an endopeptidase when incubated in the rat liver homogenate at two proteolytic cleavage sites located between Pal[3] and Ser[4], and Leu[7] and Ilys[8] sites. This result indicates that the amide bonds containing the Pal, Ser, Leu and Ilys amino acids were easily hydrolyzed by proteases which were consistent with Liao's results (Liao et al. 2008, 2010). The fragments of hydrolysis were further metabolized by hydroxylation, oxidative dechlorination, alcohol dehydration and isopropyl dealkylation reactions, and this result was in line with Reubsaet's data (Reubsaet et al. 1998a, 1998b).

The quantification of peptides in biological samples can be complicated by various degradation processes, not only in the biological matrix but also during all steps of the analytical procedure. The characterization of peptide stability and degradation is aided by recent advances in mass spectrometry (Van den Broek et al. 2008). In the present study, an LC–ESI-MS^n method was applied to identify the LHRH decapeptide fragments. In the MS^n spectrum of the decapeptide, the fragments were generated by the loss of

the H_2O, CO_2 and $CONH_2$ groups, amino acid residues and side chain, amino group and proton.

It is difficult to make an intensive investigation of peptide and protein metabolism, because they can be easily metabolized to generate innumerable molecular fragments (Muller et al. 1999; Brugos and Hochhaus 2004). Therapeutic peptides and proteins are commonly hydrophilic molecules which will be digested to a much higher extent by tissue homogenates containing large amounts of cytosolic enzymes (Liao et al. 2010). To identify the influence of these organs on enzymatic degradation, homogenates of liver or kidney can be used for enzymatic stability studies. A further in vivo study of these peptides is needed to confirm these in vitro results.

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