Hepatic in vitro metabolism of peptides; comparison of human liver S9, hepatocytes and Upcyte Hepatocytes with cyclosporine A, leuprorelin, desmopressin and cetrorelix as model compounds

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1. INTRODUCTION

Peptides are polymers of naturally occurring or artificial amino acids linked together by amide bonds. Peptide drugs have some advances over small molecule drugs. e,g, having higher affinity to target and having lower toxicity profiles[1]. The greatest downfall of peptide drugs are their short half-life and low *in vivo* stability due to quick degradation by protease enzymes that causes hydrolysis of amide bonds[2]. Therefore, metabolism (often called catabolism) of peptides is mostly non-CYP dependent and more often extrahepatic than for traditional small drugs, although some exceptions exists.

In this study, stability and metabolism of four peptide drugs were investigated in several hepatic human in vitro enzyme sources. The four peptide drugs selected for the tests were leuprorelin, cyclosporine, cetrorelix and desmopressin, which vary in structure (linear vs cyclic), and *in vivo* plasma half-life. Cetrorelix and leuprorelin have a linear structure; the approximate half-life of leuprorelin is 3h (after intravenous administration)[3] and the half-life of cetrorelix is reported to vary between 5 and 10h (after sub-cutaneous injection)[4]. Leuprorelin (also known as leuprolide) consists of nine naturally occurring amino acids with molecular weight of 1209 Da. Even though the N-terminus is ethylated, linearity and sequence consisting of natural amino acids explains short half-life via peptidase-catalyzed hydrolysis of amide bonds. Cetrorelix (MW = 1431 Da) is more modified compared to leuprorelin, which may explain better stability *in vivo*. It consists of eleven amino acids, with first four amino acids of C-terminus being synthetic amino acid-like compounds. This may affect the ability of peptidases to hydrolyze amide bonds and prolongating the stability of cetrorelix. Of the two cyclic peptides included, cyclosporine is relatively stable as half-life *in vivo* in blood varies between 6 to 20 hours.[5] Untypically to peptides, the metabolism of cyclosporine (MW = 1208 Da) is cytochrome P450 (CYP3A4)-dependent and it forms more than 30 metabolites via various metabolic reactions.[6] Unlike most of the other peptide drugs, it may be dosed orally as it is stable enough to survive the first-pass metabolism and acidic environment of gastrointestinal tract. It consists of eleven amino acids and seven of its amino acid residues are N-methylated. Not all amino acids are naturally occurring, as cyclosporine contains butenyl-methyl-threonine (Bmt), L-alpha-aminobutyric acid (Abu) and sarcosine (Sar). The structure of cyclosporine is fully cyclic. while the structure of desmopressin (MW =1069 Da) is semi-cyclic, containing disulfide-bridge between the first and sixth amino acid. It mostly consists of naturally occurring amino acids, as it is an analogue of human diuretic hormone vasopressin. Only exception is that 3-mercaptopropionic acid is used to form a disulfide bridge between cysteine's thiol-group. Cyclicity and use of synthetic amino acids are among strategies that has been implemented in design of peptide therapeutics.[7] It has also been previously noted, that N-methylation is beneficial for peptides as it enhances solubility and reduces undesired polymerization.[8]

Since metabolism of most peptide drugs is non-CYP dependent, use of only liver-derived models may be poorly-advised, as also other tissues and blood may have a high role in their metabolic clearance. However, the hepatic models, together with blood/plasma and kidney are the most regularly used systems for peptide-like drugs. Published peptide metabolism and stability studies in hepatic *in vitro*-models are relatively scarce and one goal of this study is to present new data to evaluate the comparability of these models for

peptide studies. For that, liver S9 fraction, suspended primary hepatocytes, and plated Upcyte® hepatocytes were used as models with the four peptide drugs, and UPLC/HR-MS analysis was used to evaluate the disappearance of the peptides and to detect the formed metabolites.

2. EXPERIMENTAL

2.1 Reagents and materials

HPLC grade methanol was purchased from Merck (Lichrosolv GG, Darmstadt, Germany). Acetic acid and ammonium formate were purchased from BDH Laboratory Supplies (Poole, England). Laboratory water was prepared in-house with Direct-Q water purifier (Millipore, Molsheim, France). Human liver S9-fraction, cryopreserved human primary hepatocytes, In vitro GRO HT-hepatocyte thawing medium and In vitro GRO KHB-hepatocyte incubation media were purchased from Bioreclamation IVT (Brussels, Belgium). Upcyte hepatocytes, MSE001 Hepatocyte thawing medium and MHE003 Hepatocyte high performance medium were purchased from Upcyte technologies (Hamburg, Germany). Nicotinamide adenine dinucleotide phosphate (NADPH), disodium phosphate (Na_2HPO_4,), monopotassium phosphate (KH_2PO_4), cyclosporine A, leuprorelin, desmopressin and cetrorelix were purchased from Sigma-Aldrich (Helsinki, Finland). Phosphate buffer was prepared in-house by weighing 11.35 g of Na_2HPO_4, 2.722 g of KH_2PO_4 and 0.476 g of $MgCl_2$ into a container bottle and 1 L of ultra-purified water was added to obtain 100 mM phosphate buffer.

Stock solutions (10 mM) of leuprorelin, desmopressin and cetrorelix were prepared by weighing calculated amount of peptide into glass vial and dissolving with 20% dimethylsulfoxide (DMSO) in water (v/v). Due to solubility limitation, cyclosporine A was first diluted by DMSO to obtain 10 mM stock solution; further dilution was obtained by adding 20% DMSO in MeOH (v/v).

2.2 Liver S9 fraction incubations

Human liver S9 fraction (mixed gender pool) was diluted into 100 mM phosphate buffer (pH 7.4) with 2 mM $MgCl_2$ to obtain a final enzyme protein concentration of 2 mg/ml in the incubation with a final volume of 300 µl. A 297 µl aliquot of the mixture was preincubated for 6 min at 37°C in a shaking incubator block (Eppendorf Thermomixer 5436, Hamburg, Germany) and the reaction was started by adding 3 µl of study compound (1000 µM and 100 µM in 20% DMSO), to have final test concentrations of 10 µM and 1 µM, with 0.2% DMSO. Duplicate incubations were prepared for both test concentrations. The incubations were performed with and without NADPH (1 µM). 40 µl samples were collected at 0 min, 10 min, 20 min, 40 min and 60 min timepoints. The reaction was terminated by adding two-fold volume of ice cold 75% acetonitrile in water (v/v) containing 100 nM phenacetin as an internal. Each sample was then centrifuged (13 000g, 10 min in room temperature, Heraeus Pico 17, Thermo Scientific) and the supernatant was collected for analysis.

2.3 Primary hepatocyte incubations

Vials of cryo-preserved human hepatocytes (lot ZKO) were thawed in water bath and were then suspended into InVitro GRO HT-medium (LOT C090270). After thawing, the cells were centrifuged (50 x *g*, 5 min in RT) to obtain a hepatocyte pellet. The pellet was transferred into InVitro GRO KHB-medium (lot C19017A) and was diluted so that 2 million viable hepatocytes per one milliliter cell density was obtained, determined by trypan blue test. Viability of the cells was 72%. The study compounds were preincubated in incubation media for 6 min at 37 ^{o}C at 2 µM and 20 µM concentration, and the reactions were started by mixing the cells and study compound media (1:1 v/v), to have final test compound concentrations of 1 µM and 10 µM and cell density of 1 million viable cells/ml. Incubations were prepared in duplicate at 1 µM test concentration, and in one duplicate at 10 µM test concentration. The total incubation volume was 300 µl, and samples were collected at 0 min, 10 min, 20 min, 40 min and 60 min timepoints. The reaction was terminated by addition of two-fold volume of ice cold 75% acetonitrile in water (v/v) containing 100 nM phenacetin. Each sample was then centrifuged (13 000g, 10 min) and the supernatant was collected for immediate analysis.

2.4 Upcyte hepatocyte incubations

Upcyte hepatocytes were thawed and seeded in medium containing 0.5% DMSO in collagen-coated flask to induce enzyme activity. After four days seeding period, the upcyte hepatocytes were transferred into 96-well plate at 150 000 cells/cm^2 density in medium containing 0.1% DMSO for the cells to reach full confluence in three days. The medium was replaced by 100 µl medium containing 10 and 1 µM of the study compounds, and samples were collected at 0 min, 10 min, 20 min, 40 min and 60 min. Incubations were prepared in duplicate at 1 µM test concentration, and in one duplicate at 10 µM test concentration. The reaction was terminated by collecting a 50 µl sample and adding two-fold volume of ice cold 75% acetonitrile in water (v/v) containing 100 nM phenacetin. Each sample was then centrifuged (13 000g, 10 min) and the supernatant was collected for analysis.

2.5 LC-MS analysis

Sample analysis was performed by an Acquity ultra high pressure liquid chromatography (UPLC) system (Waters Corp, Milford, MA, USA) equipped with an Acquity photo-diode-array (PDA) and Thermo Scientific Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (QE-Orbitrap-MS) (Thermo Scientific, Waltham, MA, USA). The analytical column for leuprorelin, desmopressin and cetrorelix was reversed-phase BEH C18 (2.1 x 50mm, 1.7 µm, Waters Corp) with guard filter. Eluents used were 0.1% acetic acid (A, pH 3.2) and methanol (B). The gradient elution for leuprorelin, desmopressin and cetrorelix was as follows: 2-2-10-50-95-95 B% in 0-0.5-2-3.5-7-8 min with one minute equilibration in initial conditions. The column oven temperature was 35 ^{o}C, injection volume was 4 µl and the flow rate was 500 µl/min. The used analytical column for cyclosporine was reversed-phase BEH Shield RP18 (2.1 x 50 mm, 1.7 µM, Waters Corp) with guard filter and eluents were 2 mM ammonium formate (A, pH 6.3) and methanol (B). The gradient elution for cyclosporine was as follows: 2-2-70-95-95 B% in

0-0.5-5-7-8 min with one minute of equilibration in initial conditions. The column oven temperature was 70 °C, injection volume was 4 µl and the flow rate was 500 µl/min.

The HR-MS data acquisition was performed at data-dependent-MS^2 mode which performed a full spectral scan at 35 000 resolution (FWHM @ m/z 200) and triggered further MS/MS experiments at 17 500 resolution for ions in inclusion list and also for the 3 most abundant ions not included in inclusion list. The mass range of m/z 140 - 2100 was acquired, with acquisition time of 7 Hz, maximum interval time being 100 ms. The used capillary voltage was 3000 V and capillary temperature of 320 °C was used. The auxiliary gas heater temp was set at 500 °C. Nitrogen was used as Auxiliary gas at 20 arbitrary units, Sheath gas at 50 arbitrary units and as a Sweep gas at 5 arbitrary units. Thermo Xcalibur 4.0 software was used to operate both the mass spectrometer and UPLC-system. The ion chromatograms were extracted from the total ion chromatograms using calculated monoisotopic accurate masses with 5 mDa window. The metabolites were mined from the data using software-aided data processing (Thermo Compound Discoverer 2.1, including structure-intelligent dealkylation tool & mass defect filter, Thermo Scientific) with manual confirmation.

3. RESULTS AND DISCUSSION

The disappearance of the model peptides was determined by using the 1 µM incubation concentration and 10 µM incubation concentration was used for the metabolic identification. The disappearance data was used to evaluate the half-life and intrinsic clearance of the model peptides. Summary of the observed metabolic turnover and the detected metabolites are collected to Tables 1 – 6, and the elucidated hydrolytic sites are shown in Figures 2 - 4. The chromatograms at 0 min in every studied *in vitro* system for the model peptides are presented in Figures 5 – 8.

3.1 Leuprorelin

Leuprorelin disappeared in incubations with human liver S9 fraction with half-lives of 12 min with NADPH and 35 min without NADPH, i.e. the clear NADPH-dependency in disappearance suggests involvement of CYP enzymes. The half-lives with primary hepatocytes was 44 min. No disappearance of leuprorelin was observed in the incubation with upcyte hepatocytes.

In total 13 metabolites (M1 – M13) were detected from different metabolic systems (Table 3). The greatest number of metabolites was observed with liver S9 fraction with NADPH, i.e. twelve (M2 - M13). However, only seven metabolites were detected with S9 when NADPH was not included as a cofactor for CYPs, pointing that the metabolism is partially via CYP-enzymes. M3, formed via deamidation of N-ethylated C-terminal to acid, was the very clear main metabolite in liver S9 fraction without NADPH, being almost fully in charge of the metabolic turnover, although also six other low-level metabolites were detected. On the contrary, most of the other detected metabolites were formed solely or clearly more abundantly in the presence of NADPH; suggesting involvement of CYP enzymes in their formation, which is in agreement with the clearly higher metabolic turnover of leuprorelin in the incubations with NADPH (in comparison

to without NADPH). As seen from Table 3, M3 is further metabolized to form metabolites M2, M4, M5, M7, M9 and M10, especially in liver S9 fraction, but not in similar rates in any other assays.

M3 was a clear main metabolite also with hepatocytes. The metabolite profile in primary hepatocytes was very similar to liver S9 without NADPH, i.e. in addition to M3, eight low abundance metabolites were detected, six of these being the same as with liver S9 without NADPH. Thus, surprisingly, the activation of liver S9 CYPs by NADPH had clearly an effect that natively active CYPs in hepatocytes did not show. Possible explanation to this could be slow cell uptake of leuprorelin, stressing the metabolite profile to M3 and not to its CYP-catalyzed further metabolites. If that is the case, a use of longer incubations with hepatocytes would be advised. When using upcyte hepatocytes, M3 was the main metabolite, but with only a very low abundance, accompanied by M11 and M13.

Zvereva et al. [9] determined the metabolism of various gonadotropin releasing hormone analogues, including leuprorelin, in human kidney microsomes. They detected five different metabolites formed via single hydrolysis, of which four were detected in this study. The only non-detected metabolite was formed by cleavage between fifth and sixth amino acids (Tyr-Leu), but in this study the same metabolite was detected only after the ethylation of N-terminus was removed. It could be, that they used targeted methods only, so metabolites formed via multiple hydrolysis might not have been screened. The article was inconclusive about this matter.

3.2 Cetrorelix

Cetrorelix disappeared in incubations with human liver S9 fraction with half-lives of 33 min with NADPH and 42 min without NADPH, suggesting that the observed metabolism is possibly partially CYP-dependent. Half-lives with primary hepatocytes and upcyte hepatocytes were 62 min and about 290 min, respectively.

In total six different metabolites (M1 – M6) were detected in incubations with different metabolic systems. The greatest number of metabolites was observed with liver S9 fraction with NADPH, i.e. five, while three metabolites were detected without NADPH, pointing that two oxidated low level metabolites M4 and M5 that were detected only with NADPH are formed by CYP-enzymes. Interestingly and similarly to leuprolelin, the NADPH-dependent metabolites observed with S9 were not detected with primary hepatocytes. Otherwise, formation of hydrolyzed metabolites M1 – M3 were very similar with and without NADPH, and also with primary hepatocytes. Of these, M2 (hydrolysis of amidated alanine from C-terminal) was a clear main metabolite in liver S9 fraction with and without NADPH and in primary hepatocytes, followed by M3 (hydrolysis of alanine and proline from C-terminal). M1, M2 and M3 are all products from hydrolysis of amide bonds between natural amino acids next to each other, while no hydrolysis was observed next to synthetic amino acids. With primary hepatocytes also di-hydrolysis product M6 (serine-tyrosine), which was not seen in other systems, was detected. With upcyte hepatocytes, only M2 was detected.

Both *in vitro* and *in vivo* metabolism data of cetrorelix are hard to come by, as only notable published literature is by Schwahn et al[11], who studied the metabolism of cetrorelix after single s.c. administration in rats and dogs. M1 and M2 were detected, but M3 and M6 were not. The species dependent activity of the peptidase enzymes could explain the difference, and di-hydrolysis product M6 was only detected in the hepatocytes. They also detected two single hydrolysis metabolites formed via hydrolysis between citrulline and leucine, and between serine and tyrosine from C-terminal, respectively, which were not detected in the studied *in vitro* systems.

3.3 Desmopressin

The disappearance of desmopressin in incubations with liver S9 was similar with and without NADPH, with half-lives of 43 min under both conditions, suggesting that no CYP-mediated metabolism occurs. The half-life with primary hepatocytes was 106 min, and no clear disappearance was detected in incubation with upcytes hepatocytes, as the detected peak area after 120 min incubation was 95%.

Desmopressin formed the lowest number of metabolites of the investigated peptides, as only one and the same metabolite (M1) was detected in incubations with all tested hepatic enzyme sources, even though its half-life *in vivo* in plasma is only 2 hours. Based on this, the metabolic enzymes responsible for metabolism of desmopressin are not active in these investigated enzyme sources or the cyclic structure with disulfide bond stabilizes the structure so there are no other metabolic pathways. The observed metabolite was formed via hydrolysis of proline-arginine-bond outside the cyclic part of the structure, and its abundance was similar in incubations with liver S9 with and without NADPH, which is in agreement that the metabolism of desmopressin is not mediated by CYP-enzymes. The abundance with primary hepatocytes was slightly lower than in liver S9, while only traces of the metabolites was detected with upcyte hepatocytes.

Zvereva et al [12] detected two metabolites of desmopressin in human liver S9 fraction, both resulting of the hydrolysis of the linear amino acid chain between proline and arginine, and cysteine and proline, respectively. They used lower S9 fraction concentration (0.1 mg/mL versus 2 mg/mL), and the incubation time was 24 h, compared to 1h of total incubation time of this study. Both metabolites were also detected by Esposito et al [13] who used 0.5 mg/mL S9 fraction concentration and 6 h incubation time without added cofactors. Unfortunately, kinetic formation data of the metabolites is not available. These differences might explain why one metabolite was not detected.

3.4 Cyclosporine

For cyclosporine, no clear disappearance was observed with human liver S9, with or without cofactors, or with upcyte hepatocytes. However, some disappearance was observed with primary hepatocytes, with half-life of about 111 min

In total, ten metabolites (M1 – M10) were detected from different metabolic systems. The highest number of metabolites, i.e. eight, were observed with liver S9 fraction with NADPH and hepatocytes, six of them being detected with both systems. With liver S9

fraction, all metabolites were formed completely NADPH-dependently, pointing that the metabolism of cyclosporine is fully CYP-mediated, as expected. All detected metabolites were formed via typical CYP-mediated reactions (oxidations, demethylations, dehydrogenations), with additional acetylation in M5 in hepatocytes, and hydrolytic reactions were not observed. The metabolite M6, formed via hydroxylation (oxidation), was the clear main metabolite in both systems. Based on the literature, several possible reaction sites are possible, but these were not distinguishable here with the used techniques without reference standards[14]. The abundance of the metabolites was lower in hepatocytes compared to that in liver S9 fraction. No metabolites were detected with incubation with upcyte hepatocytes.

4. CONCLUSIONS

Disappearance and metabolism of the four investigated peptides were distinct for used metabolic system. In general, S9 fraction with NADPH as a cofactor produced the highest number of metabolites and the also disappearance rates were the highest with the same system, except for cyclosporine that is fully metabolized via CYP enzymes, and metabolite formation and disappearance was compatible when primary hepatocytes were used.

Leuprorelin formed the highest number of metabolites and had the shortest half-lives of the investigated peptides in almost every enzyme source. The metabolism of leuprorelin in liver S9 was shown to be a mixture of non-CYP mediated and CYP mediated hydrolytic reactions. Interestingly, the metabolite profile observed with primary hepatocytes however correlated very well with the non-CYP metabolite profile obtained with liver S9 (without NADPH), which raises the question if the metabolism in hepatocytes is also mainly non-CYP catalyzed; or if the cell uptake rate of leuprorelin, or some other mechanism, causes the effect, However, similar results were obtained with cetrorelix, i.e. the NADPH-dependent metabolites observed with S9 were not detected with primary hepatocytes, although the metabolite profiles were otherwise similar between S9 and hepatocytes. On the other hand, the NADPH-dependent CYP catalyzed metabolism of cyclosporine was similar between liver S9 and hepatocytes. Also, the hydrolytic metabolism of desmopressin was similar between liver S9 and hepatocytes. The lack of the peptide metabolites in the upcyte hepatocytes may be caused by the short incubation time as the metabolism of peptides in the hepatic cells primarily occurs via transmembrane transporters mediated by carriers or by endocytosis and pinocytosis[15].

S9 fraction is relatively cheap and is a good alternative for in vitro metabolism studies since it contains most of the important drug metabolizing enzymes and the role of enzymes can be studied by altering the cofactors in incubation, and are not limited by cell uptake range, similarly than hepatocytes.

Figure captions

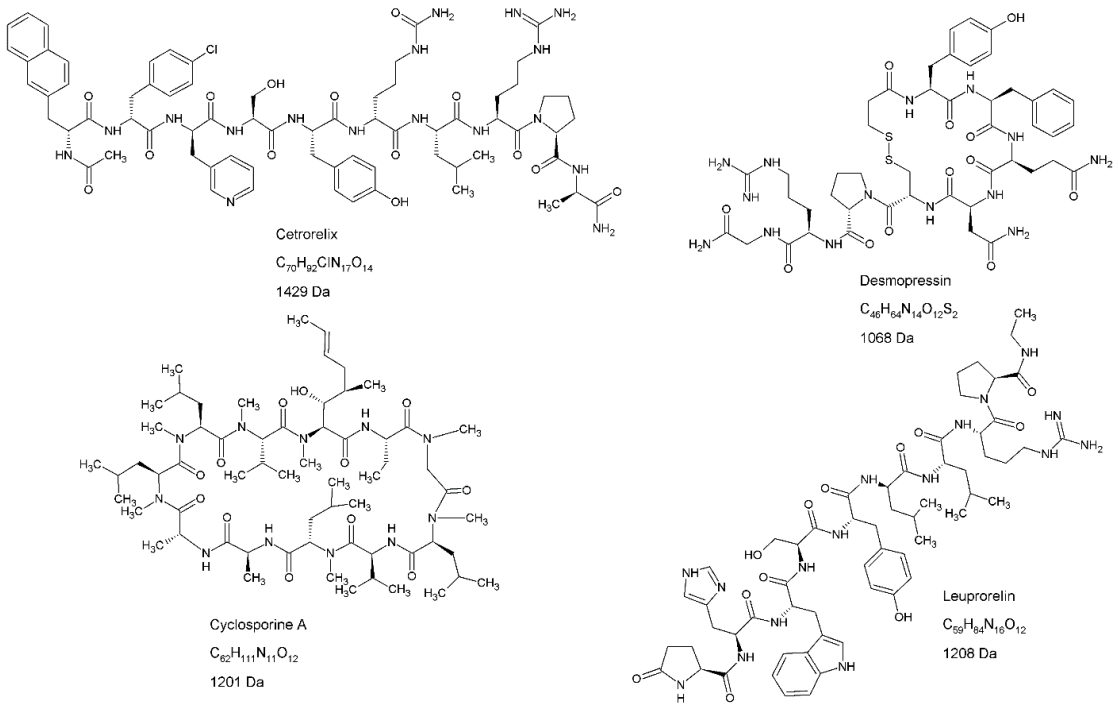

Figure 1. The structures, molecular formulas and molecular weights of the test compounds.

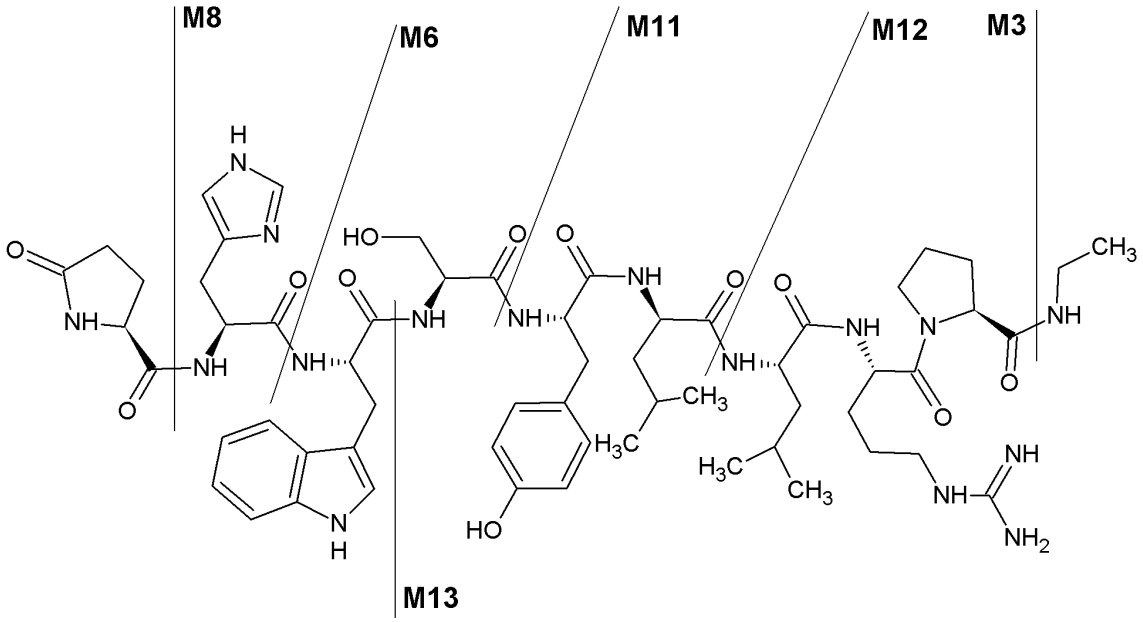

Figure 2. The hydrolysis sites for the observed hydrolytic cleavage sites for leuprorelin.

All other metabolites were formed via same reactions, by their combinations.

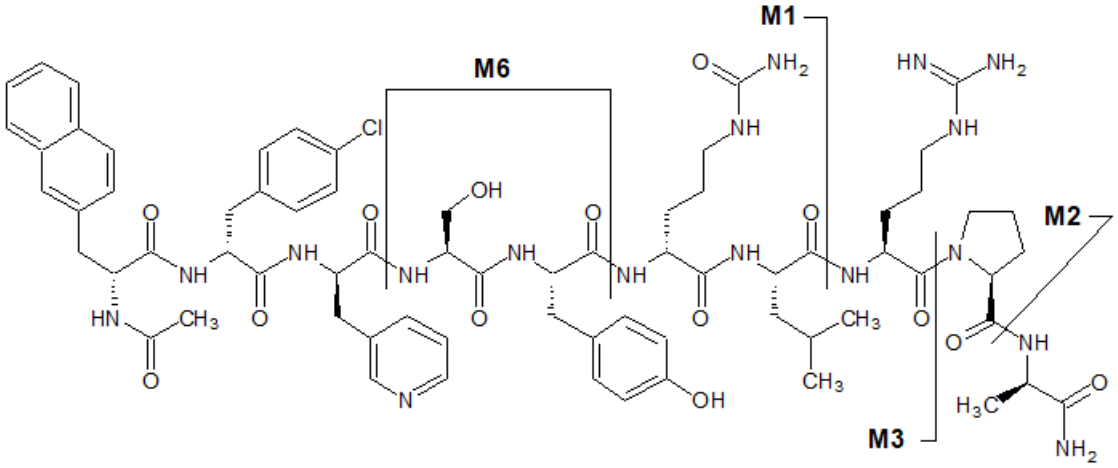

Figure 3: The hydrolysis sites for the observed hydrolytic metabolites of cetrorelix.

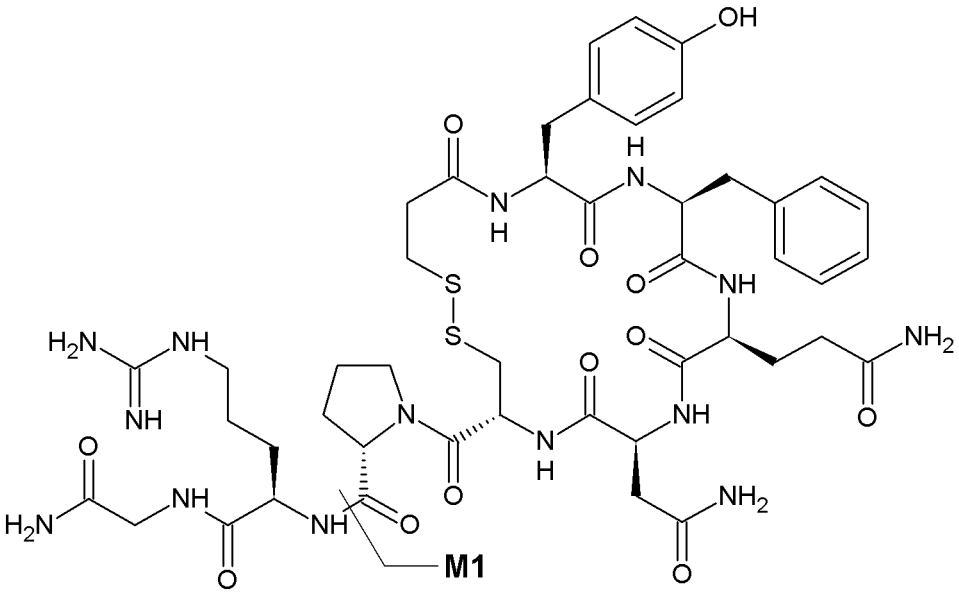

Figure 4. The hydrolysis sites for the observed hydrolytic metabolites of desmopressin.

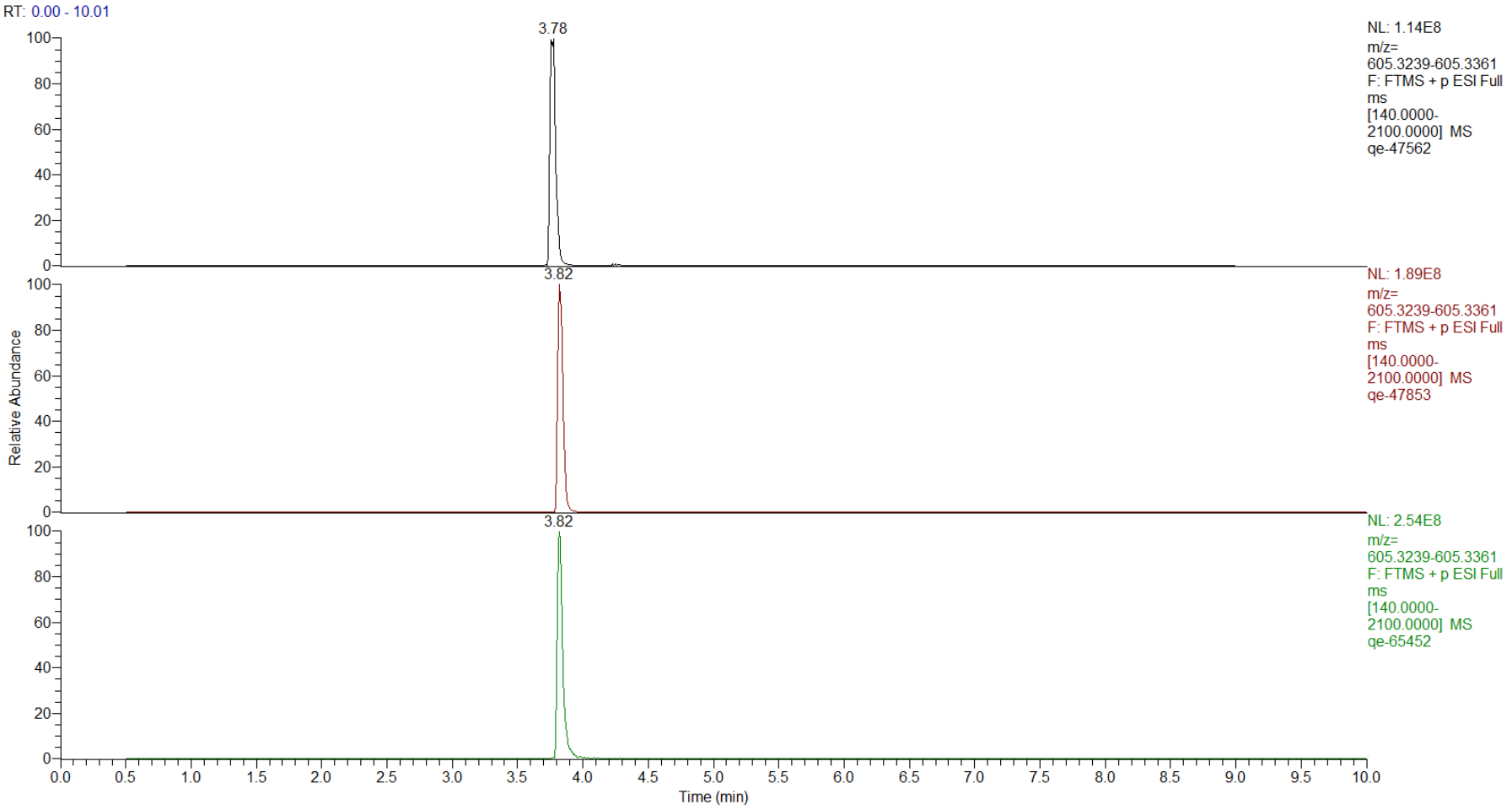

Figure 5. The chromatograms of leuprorelin at 1 µM concentration in liver S9 fraction (top), hepatocytes (middle), and upcyte hepatocytes (bottom).

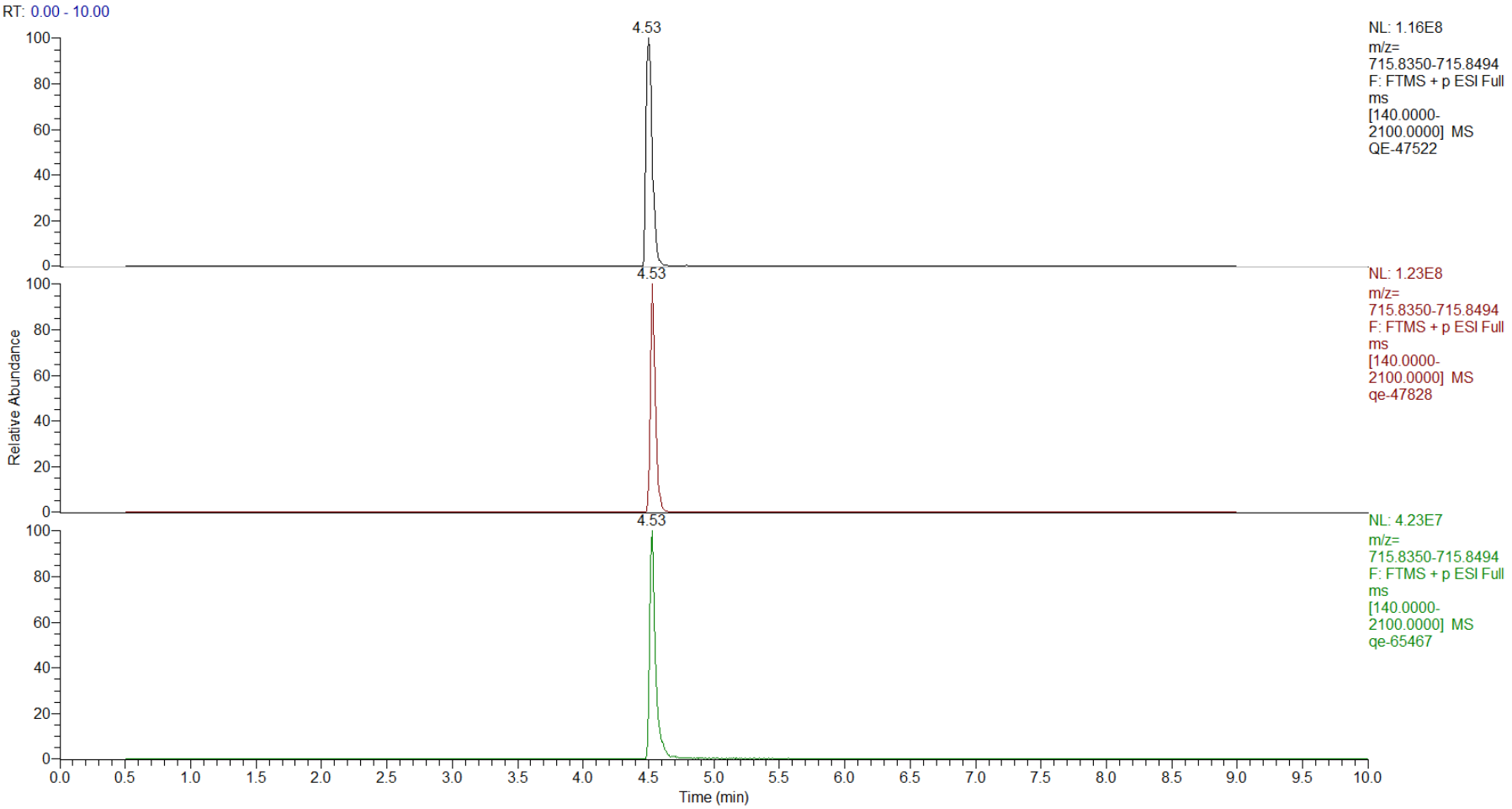

Figure 6. The chromatograms of cetrorelix at 1 µM concentration in liver S9 fraction (top), hepatocytes (middle), and upcyte hepatocytes (bottom).

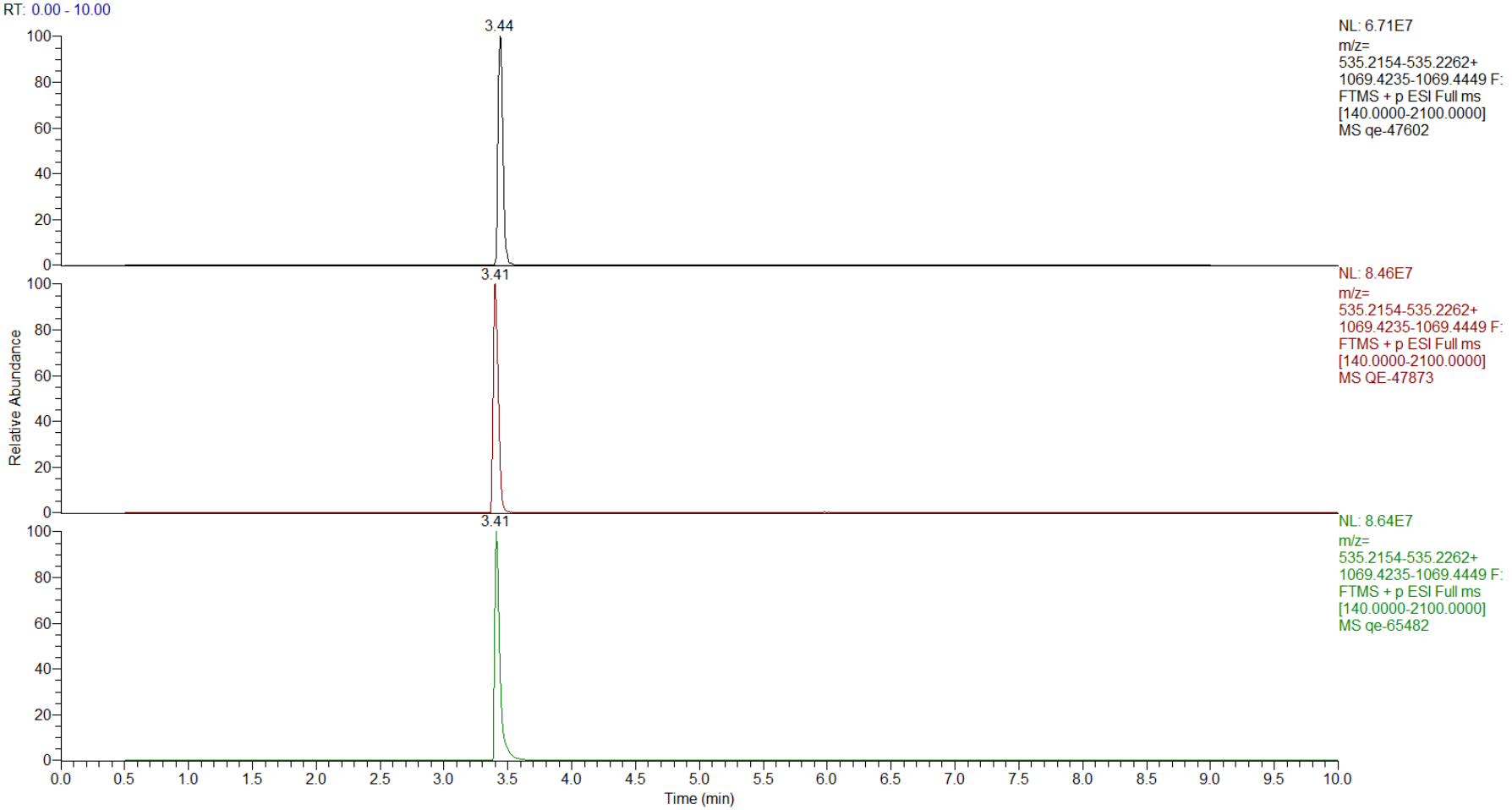

Figure 7. The chromatograms of desmopressin at 1 µM concentration in liver S9 fraction (top), hepatocytes (middle), and upcyte hepatocytes (bottom).

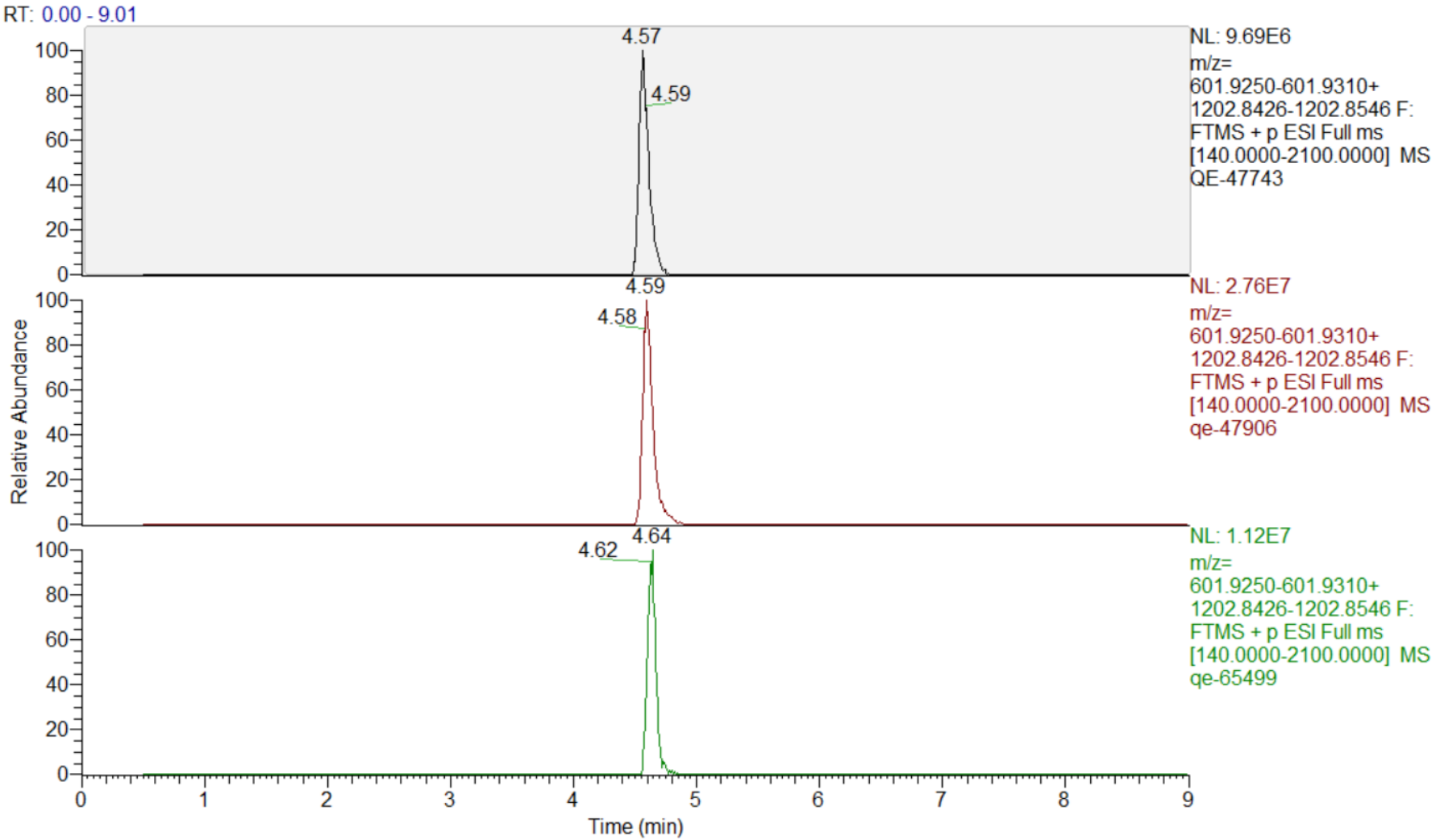

Figure 8. The chromatograms of cyclosporine at 1 µM concentration in liver S9 fraction (top), hepatocytes (middle), and upcyte hepatocytes (bottom).

Table captions

Compound	S9 with NADPH min (Cl_{int})	S9 w/o NADPH min (Cl_{int})	Hepatocytes min (Cl_{int})	Upcytes min (Cl_{int})	Medium control min
Leuprorelin	12 (27.9)	35 (9.9)	44 (15.6)	>394 (<1.8)	27
Cetrorelix	33 (10.5)	42 (8.3)	62 (11.2)	288 (4.8)	158
Desmopressin	43 (8.0)	43 (8.0)	106 (6.5)	>394(<1.8)	>394
Cyclosporine	>394 (<0.88)	>394 (<0.88)	111 (6.2)	>394(<1.8)	25

Table 1. Half-lives (min) and intrinsic clearances (in parenthesis, µl/min/mg of protein for S9, µl/min/million cells for hepatocytes/upcytes) of each peptide in each metabolic model.

Compound	S9 with NADPH 60 min	S9 w/o NADPH 60 min	Hepatocytes 60 min	Upcytes 60 min	Medium control 60 min
Leuprorelin	12	7	9	3	0
Cetrorelix	5	3	4	1	0
Desmopressin	1	1	1	1	0
Cyclosporine	8	0	8	1	0

Table 2. Number of observed metabolites for each peptide in each metabolic system.

	Name	Formula	Sequence	S9 with NADPH 60 min, %	S9 w/o NADPH 60 min, %	Hepatocytes 60 min, %	Upcytes 60 min, %
	Leuprorelin	C59H84N16O12	PyrHWSYLLRPNHEt	7.8	48.1	96.9	97.0
M1	2 x hydrolysis (aa4-aa5 + aa7-aa8)	C21H33N3O5	YLL			0.1	
M2	2 x hydrolysis (aa2-aa3 + aa9-NHEt)	C46H67N11O10	WSYLLRP	7.7	0.2	0.2	
M3	hydrolysis (aa9-NHet)	C57H79N15O13	PyrHWSYLLRP	11.0	48.2	19.1	0.7
M4	2 x hydrolysis (aa2-aa3 + aa8-aa9)	C41H60N10O9	WSYLLRP	0.1		0.5	
M5	2 x hydrolysis (aa4-aa5 + aa9-NHEt)	C32H52N8O7	YLLRP	4.1	0.3	0.3	
M6	hydrolysis (aa2-aa3)	C48H72N12O9	WSYLLRPNHEt	6.6	0.2	0.7	
M7	2 x hydrolysis (aa1-aa2 + aa9-NHEt)	C52H74N14O11	HWSYLLRP	15.2			
M8	hydrolysis (aa1-aa2)	C54H79N15O10	HWSYLLRPNHEt	9.3			
M9	2 x hydrolysis (aa3-aa4 + aa9-NHEt)	C35H57N9O9	SYLLRP	9.5	0.3	0.2	
M10	2 x hydrolysis (aa5-aa6 + aa9-NHEt))	C23H43N7O5	LLRP	0.2			
M11	hydrolysis (aa4-aa5)	C34H57N9O6	YLLRPNHEt	5.2	0.6	2.4	0.3
M12	hydrolysis (aa6-aa7)	C19H37N7O3	LRPNHEt	0.4			
M13	hydrolysis (aa3-aa4)	C37H62N10O8	SYLLRPNHEt	12.2	0.7	1.8	0.2

Table 3. Formed metabolites of leuprorelin and their percentual abundances in the end of the incubation, based on LC/MS peak area compared to parent at 0 min. Order by ascending retention time. The site of hydrolysis in parenthesis. The remaining parent percentage is different from the stability study because higher compound concentration was used.

	Name	Formula	Sequence	S9 with NADPH 60 min, %	S9 w/o NADPH 60 min, %	Hepatocytes 60 min, %	Upcytes 60 min, %
	Cetrorelix	C70H92ClN17O14	Ac-{d-A[3-(2-naphtyl)]}-[d-F(4-Cl)]-{d-A[3-(3-pyridyl)]}-SY-(D-Cit)-LRP-d-A-NH2	51.4	53.4	76.8	73.1
M1	hydrolysis (aa8-aa9)	C56H67ClN10O12	Ac-{d-A[3-(2-naphtyl)]}-[d-F(4-Cl)]-{d-A[3-(3-pyridyl)]}-SY-(D-Cit)-L	0.1	0.1	1.5	
M2	hydrolysis (aa10-aa11)	C67H86ClN15O14	Ac-{d-A[3-(2-naphtyl)]}-[d-F(4-Cl)]-{d-A[3-(3-pyridyl)]}-SY-(D-Cit)-LRP	21.5	20.8	18.3	0.5
M3	hydrolysis (aa9-aa10)	C62H80ClN14O13	Ac-{d-A[3-(2-naphtyl)]}-[d-F(4-Cl)]-{d-A[3-(3-pyridyl)]}-SY-(D-Cit)-LR	4.5	4.6	2.5	
M4	oxidation (+O)	C70H92ClN17O15	Ac-{d-A[3-(2-naphtyl)]}-[d-F(4-Cl)]-{d-A[3-(3-pyridyl)]}-SY-(D-Cit)-LRP-d-A-NH2	0.3			
M5	oxidation (+O)	C70H92ClN17O15	Ac-{d-A[3-(2-naphtyl)]}-[d-F(4-Cl)]-{d-A[3-(3-pyridyl)]}-SY-(D-Cit)-LRP-d-A-NH2	0.2			
M6	2 x hydrolysis (aa4-aa5 + aa6-aa7)	C12H14N2O5	SY			0.9	

Table 4. Formed metabolites of cetrorelix and their percentual abundances in the end of the incubation, based on LC/MS peak area compared to parent at 0 min. Order by ascending retention time. The site of hydrolysis in parenthesis. The remaining parent percentage is different from the stability study because higher compound concentration was used.

	Name	Formula	Sequence	S9 with NADPH 60 min, %	S9 w/o NADPH 60 min, %	Hepatocytes 60 min, %	Lysosomes 120 min, %	Upcytes 60 min, %
	Desmopressin	C46H64N14O12S2	c{Mpr-YFQNC}PRGNH2	51.8	47.0	103.7	93.1	96.2
M1	Hydrolysis (aa7-aa8)	C38H48N8O11S2	c{Mpr-YFQNC}P	17.1	22.7	7.1	0.1	0.1

Table 5. Formed metabolites of desmopressin and their percentual abundances in the end of the incubation, based on LC/MS peak area compared to parent at 0 min. Order by ascending retention time. The site of hydrolysis in parenthesis. The remaining parent percentage is different from the stability study because higher compound concentration was used.

	Name	Formula	Sequence	S9 with NADPH 60 min, %	S9 w/o NADPH 60 min, %	Hepatocytes 60 min, %	Upcytes 60 min, %
	Cyclosporine	C62H111N11O12	c{d-ALLVBmtAbuSarLVLA}	76.2	99.6	69.0	68.8
M1	Demethylation (-CH_2)	C61H109N11O12	c{d-ALLVBmtAbuSarLVLA}	0.5			
M2	Dehydrogenation (-H_2)	C62H109N11O12	c{d-ALLVBmtAbuSarLVLA}	0.3		0.1	
M3	Demethylation (-CH_2)	C61H109N11O12	c{d-ALLVBmtAbuSarLVLA}	2.1		1.0	
M4	Dehydrogenation + Oxidation (-H_2 +O)	C62H109N11O13	c{d-ALLVBmtAbuSarLVLA}	1.0		0.2	0.6
M5	Di-dehydrogenation + Acetylation (-H_4+C_2H_2O)	C64H109N11O13	c{d-ALLVBmtAbuSarLVLA}			0.3	
M6	Oxidation (+O)	C62H111N11O13	c{d-ALLVBmtAbuSarLVLA}	10.9		3.6	
M7	Di-demethylation + oxidation (-C_2H_4 +O)	C60H107N11O13	c{d-ALLVBmtAbuSarLVLA}			0.1	
M8	Oxidation + demethylation (-CH_2 +O)	C61H109N11O13	c{d-ALLVBmtAbuSarLVLA}	0.3		0.1	
M9	Dehydrogenation + di-oxidation (-H_2 +O_2)	C62H109N11O14	c{d-ALLVBmtAbuSarLVLA}	0.9		0.5	
M10	Di-oxidation (+O_2)	C62H111N11O14	c{d-ALLVBmtAbuSarLVLA}	0.1			

Table 6. Formed metabolites of cyclosporine and their percentual abundances in the end of the incubation, based on LC/MS peak area compared to parent at 0 min. Order by ascending retention time. The changes in structures in parenthesis. The remaining parent percentage is different from the stability study because higher compound concentration was used.

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