A Nonradioactive Approach to Investigate the Metabolism of Therapeutic Peptides by Tagging with $^{127}$I and Using Inductively-Coupled Plasma Mass Spectrometry Analysis

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ABSTRACT

The metabolic fate of adrenocorticotropic hormone (ACTH) fragment 4–10 (4–10) was evaluated following incorporation of a nonradioactive $^{127}$I-tag and with selective detection of $^{127}$I at $m/z$ 127 by inductively coupled plasma mass spectrometry (ICP-MS). $^{127}$I has all the advantages of radioactive $^{125}$I as a metabolite tracer and, together with its detection in the femtogram range, has led to a successful metabolite profiling of $^{127}$I-ACTH (4–10) in vitro. The observed metabolic stability of this peptide in tissue preparations from human plasma > kidney S9 > liver microsomes > liver cytosol, liver S9. Metabolic turnover of $^{127}$I-ACTH (4–10) was not NADPH-dependent and, together with inhibition by protease inhibitor cocktail and EDTA, is consistent with metabolism exclusively by proteases. Our preliminary studies using chemical inhibitors suggested the involvement of metalloprotease, serine peptidase, and aminopeptidase in $^{127}$I-ACTH (4–10) metabolism. The liver is the primary site of metabolic clearance of $^{127}$I-ACTH (4–10), with kidney S9 taking four times longer to produce a metabolite profile comparable to that produced by liver S9. A total of six metabolites retaining the $^{127}$I-tag was detected by ICP-MS, and their structures were elucidated using a LTQ/Orbitrap. $^{127}$I-ACTH (4–10) underwent both N- and C-terminal proteolysis to produce $^{127}$I-Phe as the major metabolite. The $^{127}$I-tag had minimal effect on the metabolic turnover and site of proteolysis of ACTH (4–10), which, together with ICP-MS providing essentially equimolar responses, suggests that the use of a $^{127}$I-tag may have general utility as an alternative to radioiodination to investigate the metabolism of peptide therapeutics.

Introduction

The high attrition rate and increasing cost of small-molecule drug development have led many pharmaceutical companies to balance their portfolio to include peptide drugs. This is fueled by the observation that the revenue from a peptide drug can be comparable to a small-molecule drug (Reichert, 2010; Badiani, 2012), yet the attrition rate during drug development is lower because of less safety issues being encountered. The medical benefit of these peptide therapeutics has made their involvement in adsorption, distribution, metabolism, and excretion (ADME) recovery to include drug-delivery system has essentially revived the research and development of peptide drugs, as illustrated by the approval of 14 peptide therapeutics by the Food and Drug Administration during the period 2009–2012 (Albericio and Kruger, 2012; Kaspar and Reichert, 2013).

The conduct of a mass balance study using radiolabeled test article to evaluate adsorption, distribution, metabolism, and excretion (ADME) efforts have been made to increase half-lives by stabilizing them against endogenous proteases via cyclization of the peptide sequence, replacing natural with unnatural amino acids, using amide bond bioisosteres, and using N or C terminus modifications (Vlieghe et al., 2010). This is a challenging task due to the broad range of proteolytic enzymes encountered, notably the aspartic, glutamic, metallo, cysteine, serine, and threonine proteases (López-Óñin and Bond, 2008). The three largest classes of proteases, namely the cysteine, metallo, and serine proteases, used cysteine, activated water molecule, and serine, respectively, in the active site for hydrolysis of the peptide bond. More detailed information on proteases can be obtained from both the degradome (http://degradome.uniow.edu) and MEROPS (http://merops.sanger.ac.uk) databases. Despite overcoming the protease-mediated metabolic instability issue, it is still extremely challenging to improve oral bioavailability due to peptide instability in the strongly acidic environment in the stomach and their poor permeability across the gastrointestinal tract. The acceptance of nonoral dosing route by patients together with paradigm change in drug discovery to include drug-delivery system has essentially revived the research and development of peptide drugs, as illustrated by the approval of 14 peptide therapeutics by the Food and Drug Administration during the period 2009–2012 (Albericio and Kruger, 2012; Kaspar and Reichert, 2013).

Part of this work was previously presented as follows: Investigation of metabolism of peptide drug by $^{127}$I-Tagging follow by detection using capillary UPLC-ICP-MS and structure elucidation using capillary/microbore LC-ESI-LTQ/Orbitrap. Heng-Keang Lim, Yuan Cao, Xi Qiu and Jose Silva. 61st American Society of Mass Spectrometry (ASMS) Conference on Mass Spectrometry and Allied Topics; 2012 June 9–13; Minneapolis, MN. dx.doi.org/10.1124/dmd.114.059774.

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characteristics is routine for small-molecule drugs and is part of the nonclinical safety evaluation package (Isin et al., 2012). Such studies were also conducted for more than 50% of the peptide drugs approved in the last decade, but were performed for understanding the ADME properties of the peptide therapeutic and less for characterizing its mass balance. This approach acknowledges the high likelihood of not achieving mass balance due to complete proteolysis of the peptide drug into its constituent amino acids, followed by incorporation of the derived amino acids into proteins synthesized de novo (Tang et al., 2004; Vugmeyster et al., 2012). Liraglutide is an example with documented recycling of the derived fatty acid fragments and amino acids into endogenous lipids and proteins, respectively (Malm-Erjefält et al., 2010), and represents the main reason that so few radiolabeled human ADME studies of peptide therapeutics have been conducted.

Radiolabelling is an important tool for studying the pharmacology and ADME of peptide drugs due to the availability of well-established radiolabelling methods for amino acids (Schumacher and Tsonomides, 1995). However, introduction of the bulky iodine atom into the peptide drug can lead to a decreased affinity for its binding site(s). It is important to establish that radiolabelling has little or no effect on the biologic activity of the peptide drug (Loot et al., 2005; De Spiegeler et al., 2012). It is not surprising that radiolabelling is the method of choice in early discovery because of its low cost and short time for labeling in addition to high sensitivity of detection. However, 125I-based tracer has many drawbacks, such as the short half-life of 60 days (Schumacher and Tsonomides, 1995), greater radiation hazard from its decay to the more energetic gamma rays (Schumacher and Tsonomides, 1995), thyroid toxicity from selective uptake of the radioactive 125I into the thyroid gland (Jung et al., 2006), and reutilization of radiolabeled amino acid in the body pool (Tang et al., 2004), which limits its use as a tracer in humans. This has led to the development of alternative tracers for investigating the ADME properties of peptide drug across species, including humans, but without the radiation safety liability.

This work describes the development of a 127I-based metabolic tracer for metabolic profiling of peptide therapeutic in vitro using capillary ultra-performance liquid chromatography (UPLC) with detection of 11C by inductively-coupled plasma mass spectrometry (ICP-MS). The sensitivity of detection of 11C by capillary UPLC-ICP-MS analysis is established using on-column injection of a 127I-tagged peptide. The metabolic profiling process involves initial detection of the peptide drug and its metabolites containing the 127I-tag using ICP-MS, followed by reanalysis of samples using microbore ultra-high performance liquid chromatography (UHPLC) interfaced to a LTQ/Orbitrap followed by reanalysis of samples using microbore ultra-high performance liquid chromatography (UHPLC) interfaced to a LTQ/Orbitrap. The metabolite profiling process involves initial detection of the peptide drug and its metabolites containing the 127I-tag using ICP-MS, followed by reanalysis of samples using microbore ultra-high performance liquid chromatography (UHPLC) interfaced to a LTQ/Orbitrap followed by reanalysis of samples using microbore ultra-high performance liquid chromatography (UHPLC) interfaced to a LTQ/Orbitrap.

In Vitro Metabolism. All incubation, 0.5 mL total volume in 1.5 mL polypropylene microcentrifuge tube, consisted of 1 mg/mL human liver or kidney subcellular fraction (human liver S9, microsomes, or cytosol; human kidney S9) or human plasma, 10 μM 127I-ACTH (4–10), 127I-ACTH (4–10): ACTH (4–10) [5:10 μM], ACTH (4–10), 127I-ACTH (4–8), 127I-ACTH (5–10), or 127I-ACTH (7–10), and 0.1 M phosphate buffer, pH 7.4. Stock solutions of 127I-ACTH (4–10), ACTH (4–10) [10.5 μM], ACTH (4–10), 127I-ACTH (4–8), 127I-ACTH (5–10), and 127I-ACTH (7–10) were prepared in 50% of 0.1% (v/v) formic acid in methanol and serially diluted in 0.1% (v/v) formic acid prior to spiking to keep the final methanol content to 0.1%. The effect of 127I-tag on the site of metabolism was assessed from incubation of 127I-ACTH (4–10) (5.10 μM) in human liver S9. The requirement of NADPH for metabolism was investigated by adapting previously published procedure for human liver microsomal system (Lim et al., 2007) to include EDTA, MgCl2, and NADPH in above incubation mixture. An incubation was also conducted at 1 μM 127I-ACTH (4–10) to assess sensitivity of detection by the capillary UPLC-ICP-MS system. The incubation was terminated at 0 and after 15 minutes at 37°C by the addition of 30 μL 70% perchloric acid, and the tube was vortexed prior to centrifugation at 20,000g for 15 minutes at 4°C. The supernatant was then filtered by centrifugation through a prewashed 0.45-μm nylon filter at 14,000g for 5 minutes at 4°C. The filtrate was transferred to 300 μL polypropylene microvial for analysis by capillary UPLC-ICP-MS or 96-well plate for analysis by microbore UHPLC-LTQ-Orbitrap.

The turnover of ACTH (4–10) and 127I-ACTH (4–10) was investigated from duplicate incubations of 127I-ACTH (4–10): ACTH (4–10) (5.10 μM) in human liver S9 for 0 and 15 minutes. The peak area corresponding to the exact m/z of the doubly-charged ion (base peak) of ACTH (4–10) at 15-minute incubation normalized to that from 0 minute was used for calculation of the turnover of ACTH (4–10) by LTQ/Orbitrap analysis. Similarly, the peak area corresponding to 127I-ACTH (4–10) in the 127I-reconstructed ion chromatogram (RIC) at 15-minute incubation normalized to that from 0 minute was used for calculation of the turnover of 127I-ACTH (4–10) by ICP-MS analysis.

Effect of Protease Inhibitor. Incubations were conducted, as described above, in the presence and absence of 6 mM AEBSF·HCl, 4.8 μM aprotinin, 300 μM bestatin, 90 μM E-64, 120 μM leupeptin, 60 μM pepstatin A, a 16.6× final concentration of Halt protease inhibitor cocktail or a 16.6× final concentration of Halt protease inhibitor cocktail, and 2 mM EDTA, which contained the same concentrations of all previously mentioned individual protease inhibitors. The tubes were precultivated at 37°C for 10 minutes with protease inhibitor or equal volume of vehicle prior to addition of 10 μM 127I-ACTH (4–10) for further incubation at 37°C for another 15 minutes. An identical set was terminated at 0 minute for calculation of percentage of turnover. Further sample processing and analysis were as described above.

Capillary UPLC-ICP-MS. The capillary UPLC-ICP-MS used for all analyses consisted of a Waters nanoAcquity UPLC (Milford, MA) coupled to an Agilent 7700× ICP-MS (Tokyo, Japan). A total consumption DS-5 microflow concentric nebulizer inserted directly into a low-volume (8-mL) single pass spray chamber with additional gas port and connected directly via a glass socket to the base of the ICP-MS 2.5 mm ID quartz torch was used for hyphenation of capillary UPLC to ICP-MS. The column used was a ZORBAX 300SB-C18 capillary column (150 × 0.3 mm id, 3.5 μm; Agilent Technologies, Santa Clara, CA) equipped with a ZORBAX 300SB-C18 guard column (5 × 0.5 mm id, 5 μm) and kept at room temperature during analysis. All connections from capillary UPLC to ICP-MS used the shortest 1/16-inch outer diameter (OD) and 0.0025-inch ID PEEK tubings and zero dead volume fittings. The separation of 127I-ACTH (4–10) and its metabolites used a linear gradient started at 2% B for 1 minute, increased linearly to 60% B over 30 minutes, and held for 1 minute before another linear increased to 90% B over 2 minutes before returning back to 2% B in 1 minute with total run time of 45 minutes. Solvents A and B corresponded to water and methanol, respectively.
containing 0.1% (v/v) TFA and acetonitrile/methanol (1:1) containing 0.025% (v/v) TFA. The flow rate was set at 5 μL/min; the entire eluant was sprayed into the chamber using argon introduced via the carrier gas inlet at 0.85 L/min; and further improvement in aerosol transport was aided by make-up gas consisting of 20% oxygen in argon introduced at additional gas port in the spray chamber at setting of 30%. This is equivalent to doping the argon plasma with oxygen to a final amount of 6%. An Agilent 7800× ICP-MS was fitted with a nickel sampling and skimmer cones and optimized automatically in collision/reaction cell (CRC) mode off via the MassHunter software for RF power (1500 W) and the torch position (torch-H: 0.1 mm; torch-V: 1.0 mm) by infusion of 10 μL/L tuning solution at 5 μL/min, whereas the sampling depth (8.5 mm), and gas flow like argon plasma gas flow (argon: 15.0 L/min), carrier gas flow (argon: 8.0 L/min), and option gas flow (20% oxygen in argon: 30%) were optimized manually. The ICP-MS was further optimized manually for detection of I+ at m/z 127 with scan time of 1 second, and by infusion of 1 pmol/L 127I-ACTH (4–10) in 50% 0.1% (v/v) formic acid in methanol. Furthermore, the contribution of chemical background such as polyatomic ion like 40Ar2Cl+ with capillary UPLC online by collision with He in the CRC at 1.2 mL/min and using 4 V kinetic energy discrimination. Data reduction and analysis were carried out using the MassHunter software.

**Microbore UHPLC-LTQ/Orbitrap.** All microbore UHPLC-MS analyses were carried out using a Surveyor MS Plus UHPLC (Thermo Fisher Scientific, San Jose, CA) coupled to a LTQ Orbitrap (Thermo Fisher Scientific, Bremen, Germany). Chromatographic separation was conducted using a ZORBAX 350SB-C18 microbore column (150 × 0.5 mm ID, 3.5 μm; Agilent Technologies, Santa Clara, CA) at room temperature. The chromatographic separations of 127I-ACTH (4–10) and its metabolites were carried out using an liquid chromatography (LC) gradient and mobile phases, as described above, except for the flow rate set at 25 μL/min. The mass spectrometer was operated in the positive electrospray ionization (ESI) mode and with the ESI sprayer set at position D. The entire eluant was sprayed into the mass spectrometer at 3 kV with nebulizer, sheath, and sweep gas set at 45, 5, and 5 arbitrary units, respectively, and desolvation of the solvent droplets was further aided by a heated capillary temperature of 275°C.

The 127I-ACTH (4–10) and its metabolites detected by ICP-MS were identified using LTQ/Orbitrap by modification of the parent list–triggered data-dependent multiple-stage mass analysis method described elsewhere (Lim et al., 2007). Briefly, 127I-ACTH (4–10) and its metabolites were initially detected by full-scan mass analysis from m/z 100 to 1500 at a resolving power of 30,000 (at m/z 400, full width at half maximum [FWHM]) and followed by data-dependent multiple stage mass analysis at a resolving power of 7500, which was triggered by the most abundant ion from a parent list of doubly- and singly-charged ions of predicted metabolites. The accurate mass measurement which was triggered by the most abundant ion from a parent list of doubly- and singly-charged ions of predicted metabolites. The accurate mass measurement was carried out under internal mass calibration mode, as previously reported (Lim et al., 2011), except for reducing the flow rate of the internal mass calibrant to 10 μL/min into the ESI source. CID was conducted with an isolation width of 2 Da, normalized collision energy of 28% for both MS2 and MS3, respectively, activation q of 0.25, and an activation time of 30 ms. Data acquisition and reduction were carried out using Xcalibur version 2.0 (Thermo Fisher Scientific, Bremen, Germany). In addition, data were mined for the presence of 127I-ACTH (4–10)–related materials by differential analysis in MsXelerator software (MsMetrix, Maarsen, The Netherlands).

**Results**

Hyphenation of Capillary UPLC to ICP-MS. The initial attempt to hyphenate capillary UPLC to ICP-MS used a DS-5 total consumption microflow nebulizer fitted directly to a low-volume (8-mL) spray chamber (CETAC Technologies, Omaha, NE). Such configuration gave a rapid decline in sensitivity from repeated injection and column of 1 ng 127I-ACTH (4–10) under reverse-phase chromatographic separation, using a linear gradient elution from 2 to 90% mobile phase B, even down to as low as 1 μL/min flow rate. Many attempts were made to improve the stability of performance and finally settled on introduction of a final concentration of 6% oxygen to the argon plasma, which gave a greater stability in performance, but without the undesirable formation of iodine oxide (127I16O+) at m/z 143. As a result, a limit of detection from injection on-column of 4.4 pg (or 4.0 fmol) for 127I-ACTH (4–10) was observed for 127I-ACTH (4–10) with a S/N ≥ 3 and corresponded to 509.3 fg 127I (Fig. 1). Furthermore, injection on-column of 21.8 pg 127I-ACTH (4–10), corresponding to 2.5 pg 127I gave a peak area that was 4.9-fold greater than that obtained from injecting 509.3 fg 127I on-column and, therefore, established that the ICP-MS response was directly proportional to the amount of 127I injected on-column. In addition to sensitivity of detection, the current hyphenation interface resulted in a more robust operation, as indicated by continued usage of the ICP-MS for ~1 month without cleaning the nickel sampling and skimmer cones.

Fig. 1. 127I-TIC showing the limit of detection of 127I by the capillary UPLC-ICP-MS system, which corresponded to 4.36 pg (4.00 fmol) 127I-ACTH (4–10) or 509 fg 127I injected on-column. Injection on-column of 21.8 pg (20.0 fmol) 127I-ACTH (4–10) or 2.50 pg 127I injected gave the expected fivefold increase in peak area. The chromatographic separation was conducted as described in section on capillary UPLC-ICP-MS.
of peptide-derived material. The remaining metabolites (M1, M3–6) individually ranged from ~1 to 15%. The high sensitivity in detection of 127I+ by capillary UPLC-ICP-MS permitted detection of 127I-ACTH (4–10) and only four of its metabolites (M2, M4–6) from incubation of 1 µM 127I-ACTH (4–10) in human liver S9 for 15 minutes (data not shown).

The replacement of human liver S9 with cytosol gave a similar metabolite profile from turnover of ~96% of 127I-ACTH (4–10) (Fig. 2B), but replacement with liver microsomes (Fig. 2C) gave a different metabolite profile from a lower turnover of 76.0% compared with S9 or cytosol. In this case, a total of five metabolite peaks (M2–6) was detected with different relative abundances compared with S9 or cytosol (Table 1). The major metabolite in the microsomal incubation was M4 (~32%) instead of M2 (~18%). Furthermore, there was ~twofold increase in the formation of metabolite M3 by microsomes compared with S9, which was beneficial for structural elucidation of this metabolite by LTQ/Orbitrap. In addition to human liver subcellular fractions, the in vitro metabolism of 127I-ACTH (4–10) was also evaluated using human kidney S9, and there was ~26% turnover following a 15-minute incubation compared with comparable turnover

Table 1

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Liver S9 15 minutes</th>
<th>Liver Cytosol 15 minutes</th>
<th>Liver Microsomes 15 minutes</th>
<th>Kidney S9 15 minutes</th>
<th>Kidney S9 60 minutes</th>
<th>Plasma 4 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-ACTH (4–10)</td>
<td>10.2</td>
<td>8.6</td>
<td>34.1</td>
<td>84.9</td>
<td>10.9</td>
<td>95.4</td>
</tr>
<tr>
<td>M6</td>
<td>12.6</td>
<td>18.1</td>
<td>10.0</td>
<td>ND</td>
<td>6.9</td>
<td>ND</td>
</tr>
<tr>
<td>M5</td>
<td>9.3</td>
<td>8.6</td>
<td>4.9</td>
<td>2.6</td>
<td>15.3</td>
<td>ND</td>
</tr>
<tr>
<td>M4</td>
<td>15.2</td>
<td>12.7</td>
<td>31.5</td>
<td>12.6</td>
<td>9.0</td>
<td>8.6</td>
</tr>
<tr>
<td>M3</td>
<td>0.8</td>
<td>1.1</td>
<td>1.8</td>
<td>ND</td>
<td>1.3</td>
<td>ND</td>
</tr>
<tr>
<td>M2</td>
<td>51.2</td>
<td>50.1</td>
<td>17.7</td>
<td>ND</td>
<td>55.1</td>
<td>ND</td>
</tr>
<tr>
<td>M1</td>
<td>0.7</td>
<td>0.9</td>
<td>ND</td>
<td>ND</td>
<td>1.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not detected.
increased the formation of M1 (1.2-fold). Conversely, bestatin inhibited the effect on metabolites M3 and M4. However, there was comparable inhibition of proteolysis of 127I-ACTH (4–10) in human liver S9 by AEBSF or bestatin, as indicated by ~27% of 127I-ACTH (4–10) in each incubate. Surprisingly, incubation with both AEBSF and bestatin did not produce comparable inhibition of the in vitro metabolism of 127I-ACTH (4–10) as that by protease inhibitor cocktail, as indicated by detection of ~37% 127I-ACTH (4–10) (Table 2). M1 (~33%) instead of M5 was the major in vitro metabolite. Also, there was ~12% each of metabolites M5 and M6. The remaining metabolites M2, M3, and M4 accounted for ~2%, ~1%, and ~3%, respectively. Interestingly, M2 was also the major metabolite from further incubation of 10 μM each of metabolites M3, M4, and M6 in human liver S9 for 15 minutes based on contribution of 90.8%, 96.3%, and 99.5%, respectively, to percentage of composition in each incubation (Supplemental Table 1). In addition, M1 (<1%), M5 (~2%), and M6 (~1%) were also formed from incubation with M4. There was ~1% of M1 detected from incubation with M3 and much less than 1% from M6.

Effect of Protease Inhibitor on In Vitro Metabolism. The turnover of 127I-ACTH (4–10) in human liver S9 and its metabolite profile were not affected by omission of NADPH (data not shown). However, the addition of Halt protease inhibitor cocktail led to ~41% reduction in turnover compared with an incubation without protease inhibitors (compare Figs. 2A and 3A). There was incomplete inhibition of proteolysis, as indicated by ~47% increase in contribution of unchanged peptide to the percentage of composition (Table 2) and resulted in M5 (37.2%) instead of M2 as the major in vitro metabolite. All of the previously detected metabolites except M2 were also present, but each metabolite accounted for less than ~4% of 127I-ACTH (4–10)–derived materials. Interestingly, the addition of 2 mM EDTA together with the protease inhibitor cocktail led to a near-complete inhibition of proteolysis, as indicated by ~93% of 127I-ACTH (4–10) (Fig. 3B; Table 2), and the balance consisted of the minor metabolites M4–6. Further incubation of individual protease inhibitor from the Halt protease inhibitor cocktail showed that aprotinin, E-64, leupeptin, or pepstatin A had negligible inhibition on proteolysis of 127I-ACTH (4–10) in human liver S9 (data not shown). In addition to inhibition of substrate turnover, AEBSF and bestatin also affected the quantitative formation of metabolites M1 to M6 (Table 2) and illustrated by a difference in appearance of the respective 127I-RIC in Fig. 3, C and D, compared with that without protease inhibitor cocktail and EDTA in Fig. 2A. AEBSF inhibited the formation of M2 and M6 by ~twofold and increased the formation of metabolites M1 (2.6-fold) and M5 (2.4-fold) by comparable magnitude, whereas there was little effect on metabolites M3 and M4. Conversely, bestatin inhibited the formation of metabolites M2 (~ninefold) and M4 (~fivefold) and increased the formation of M1 (~38-fold) and M6 (~twofold), whereas there was little effect on metabolites M3 and M5. It appears that bestatin had the most profound inhibition on the in vitro proteolysis of 127I-ACTH (4–10) and resulted in M1 and M6 replacing M2 as the major in vitro metabolites. However, there was comparable inhibition of proteolysis of 127I-ACTH (4–10) in human liver S9 by AEBSF or bestatin, as indicated by ~27% of 127I-ACTH (4–10) in each incubate. Surprisingly, incubation with both AEBSF and bestatin did not produce comparable inhibition of the in vitro metabolism of 127I-ACTH (4–10) as that by protease inhibitor cocktail, as indicated by detection of ~37% 127I-ACTH (4–10) (Table 2). M1 (~33%) instead of M5 was the major in vitro metabolite. Also, there was ~12% each of metabolites M5 and M6. The remaining metabolites M2, M3, and M4 accounted for ~2%, ~1%, and ~3%, respectively. Interestingly, M2 was also the major metabolite from further incubation of 10 μM each of metabolites M3, M4, and M6 in human liver S9 for 15 minutes based on contribution of 90.8%, 96.3%, and 99.5%, respectively, to percentage of composition in each incubation (Supplemental Table 1). In addition, M1 (<1%), M5 (~2%), and M6 (~1%) were also formed from incubation with M4. There was ~1% of M1 detected from incubation with M3 and much less than 1% from M6.

Elucidation of Structures of 127I-ACTH (4–10) and Its Metabolites. The drug-related materials from incubation of 10 μM 127I-ACTH (4–10) or ACTH (4–10); 127I-ACTH (4–10) (10:5 μM) in human liver S9 were detected by differential analysis using MxXelerator software and from comparison of relative retention time of metabolites detected by ICP-MS and LTQ/Orbitrap analyses. In general, there was good agreement in the relative retention times of 127I-ACTH (4–10) and its metabolites detected by ICP-MS and LTQ/Orbitrap analyses, which facilitated their identifications using the LTQ/Orbitrap. Unless otherwise indicated, the sample from incubation at ASPET Journals on April 18, 2017 dmd.aspetjournals.org Downloaded from
for each detected drug-derived material, as tabulated in Table 3. The assignment of product ions was adapted with slight modification of the reported nomenclature for primary and secondary series of product ions (Biemann, 1990) and supported by chemical formulae from accurate mass measurements, as shown in Supplemental Fig. 1.

127I-ACTH (4–10). The peak at 29.02 minutes in base peak RIC in Fig. 4B was identified as the unchanged peptide based on its identical chemical formula to 127I-ACTH (4–10) provided by the protonated molecule at m/z 1088.32730 (C44H59O10N13IS, 21.5 rings plus double bonds (RDB), 0.48 ppm) and the base peak corresponding to the doubly-charged ion at m/z 544.66697 (C44H60O10N13IS, 21.0 RDB, 0.10 ppm). Collision-induced dissociation (CID) of the doubly-charged ion gave N-terminal b-series ions (b^2+, b^3+, b^5+, b^6^2+) and C-terminal y-series ions (y^3+, y^4+, y^5+, y^5^2+, y^6+, and y^6^2+), as shown in Table 3 and Supplemental Fig. 1. The b- and y-series ions together provided adequate coverage of the primary sequence to corroborate its identification as 127I-ACTH (4–10). Confirmation of its identification is from good agreement of its product ion mass spectrum with that of reference standard of 127I-ACTH (4–10) (data not shown) and also from good agreement with its retention time (Fig. 5). The previous 127I-TIC in Fig. 2A is displayed without truncation as upright 127I-TIC in Fig. 5 to show the presence of three unknown 127I-containing endogenous peaks at 3.90, 7.12, and 8.57 minutes following incubation of 10 μM 127I-ACTH (4–10) in human liver S9. The first two peaks were present in lower abundance in human liver cytosolic or microsomal incubations and only detectable at trace levels in incubations with either human kidney S9 or human plasma (data not shown).

M1. The minor metabolite M1 has a retention time relative to 127I-ACTH (4–10) of 0.64 in the 127I-RIC (Fig. 2A) and base peak RIC (Fig. 4B), which facilitated the identification of its protonated molecule at m/z 448.08405 as the base peak in the full-scan mass spectrum and generated a chemical formula of C15H23O3N5I (6.5 RDB, 0.09 ppm). The chemical formula gave a postulated dipeptide structure of 127I-Phe-Arg, and, unfortunately, no product ion mass spectrum was obtained due to its formation in trace amounts in the S9 incubate (Table 1). Instead, the higher amount of M1 in S9 incubate with added bestatin (Table 2) was used for the structure elucidation of M1 by CID and produced the product ions in Table 3. CID of the protonated molecule of M1 resulted in y^1+ and z^1+ ions, which supported arginine as the C terminus, and the a1+ ion suggested the N terminus corresponding to the 127I-phenylalanine (Supplemental Fig. 1). Together these ions corroborated M1 has a dipeptide structure of 127I-Phe-Arg.

M2. The identification of the major in vitro metabolite M2 in the 127I-RIC in Fig. 2A is facilitated by its similar retention time relative to 127I-ACTH (4–10) of 0.71 in the base peak RIC in Fig. 4B. This led to the assignment of the base peak at m/z 291.98304 in the full-scan mass

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Liver S9</th>
<th>S9 + PIC</th>
<th>S9 + PIC + EDTA</th>
<th>S9 + AEBSF</th>
<th>S9 + Bestatin</th>
<th>S9 + AEBSF + Bestatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-ACTH (4–10)</td>
<td>10.2</td>
<td>56.8</td>
<td>92.8</td>
<td>27.3</td>
<td>27.4</td>
<td>36.7</td>
</tr>
<tr>
<td>M6</td>
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<td>1.0</td>
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<td>8.2</td>
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</tr>
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<td>22.2</td>
<td>7.9</td>
<td>12.0</td>
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<td>1.3</td>
</tr>
<tr>
<td>M2</td>
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<td>ND</td>
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<td>5.5</td>
<td>2.4</td>
</tr>
<tr>
<td>M1</td>
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<td>3.5</td>
<td>ND</td>
<td>1.8</td>
<td>26.7</td>
<td>32.9</td>
</tr>
</tbody>
</table>

ND, not detected; PIC, protease inhibitor cocktail.

Fig. 4. Base peak RIC from parent list triggered high resolution accurate mass analysis of incubation of 10.5 μM ACTH (4–10)/127I-ACTH (4–10) in human liver S9 at 37°C for (A) 0 minute and (B) 15 minutes. The elution order of the detected metabolites M1–6 and the corresponding noniodinated metabolites M1’–6’ is illustrated in the base peak RIC.
The metabolites M2, M3, M4, M6, and \(^{127}\)I-ACTH (4–10) detected by capillary UPLC-ICP-MS analysis of an incubation of 10 \(\mu\)M \(^{127}\)I-ACTH (4–10) in human liver S9 (upright) were confirmed by matching retention times with authentic reference standards (inverted) in addition to comparison of their product ion mass spectra (data not shown). The retention times of reference standards were established by on-column injection of 1 \(\mu\)L human liver S9 extract containing each ~500 pg \(^{127}\)I-ACTH (4–10), M2, M3, M4, or M6.
gave doubly-charged product ions at m/z 470.14172 and 461.13714 from sequential loss of a neutral H2O molecule (Table 3; Supplemental Fig. 1). Also, CID gave structurally informative C-terminal ions such as y-series ions (y2+, y3+, y4+, and y5+) and a z+ ion. However, the N-terminal ions detected include predominantly the b-series ions (b2+, [b2-H2O]+, [b2-H2O]+, [b2-H2O]+, b3+, and [b2-H2O]+) and an a-series ion such as [a2-H2O]+ ion (Supplemental Fig. 1). The entire primary sequence of the metabolite M4 can be accounted for by the observed N- and C-terminal ions and corroborated earlier assignment of 127I-ACTH (5–10) for M4. This was confirmed by good agreement of the product ion mass spectrum with that of the reference standard 127I-ACTH (5–10) (data not shown), and also included good agreement of its retention time (Fig. 5).

M5. The metabolite M5 was separated from M4 by ~14 seconds, as reflected by its retention time relative to 127I-ACTH (4–10) of 0.96 in 127I-RIC (Fig. 2A) and base peak-RIC (Fig. 4B). This led to the identification of the protonated molecule and doubly-charged base peak ion at m/z 828.24416 and 416.62547, which gave a chemical formula of C34H43O6N11I (18.5 RDB, 0.56 ppm) and C34H44O6N11I (18.0 RDB, −0.04 ppm), respectively. The chemical formulae supported a proposed structure of 127I-ACTH (6–10) for M5. CID of the doubly-charged ion gave the C-terminal ions such as y2+, z2+, and y3+, as shown in Table 3 and Supplemental Fig. 1. In addition, CID resulted in the formation of the N-terminal ions, which included the b-series ions such as b2+, b3+, and [b2-NH3]+, and an a2+ ion. The complementary b- and y-series ions corroborated the assignment of the structure of 127I-ACTH (6–10) for M5. The confirmation of M5 is from good agreement of its product ion mass spectrum with that from the reference standard 127I-ACTH (6–10) and also from good agreement of its retention time (Fig. 5).

M6. The metabolite M6 eluted at a retention time relative to 127I-ACTH (4–10) of 0.98 in both 127I-RIC (Fig. 2A) and base peak RIC (Fig. 4B), which led to the identification of the protonated molecule and doubly-charged base peak ion at m/z 691.18505 (C28H36O5N8I, 14.5 RDB, 0.38 ppm) and doubly-charged ion at m/z 346.09533 (C28H37O5N8I, 14.0 RDB, −2.03 ppm). CID of the protonated molecule resulted in the product ions at m/z 674.15976 and 656.14785 from the sequential loss of neutral NH3 and H2O molecules, respectively. Furthermore, CID resulted in the formation of the C-terminal (y2+, z2+, and z3+) and N-terminal (b2+, [b2-NH3]+, [b2-NH3CNHNH2]+, b3+, and [b2-NH3]+) ions as displayed in Table 3 and Supplemental Fig. 1. The N- and C-terminal ions provided coverage of the entire primary sequence of 127I-ACTH (7–10) and corroborated the above structural assignment of M6. The good agreement of the product ion mass spectrum of M6 with that of the reference standard 127I-ACTH (7–10) (data not shown) together with good agreement of its retention time (Fig. 5) confirmed the assignment of the structure of 127I-ACTH (7–10) for M6.

Interestingly, no ACTH (4–10) or noniodinated metabolites were detected from incubation of 10 μM 127I-ACTH (4–10) in human liver S9 (data not shown). However, the corresponding noniodinated metabolites were detected and labeled M1–6 following incubation of ACTH (4–10): 127I-ACTH (4–10) (10.5 μM) in human liver S9, as depicted in Fig. 4B. The detection of each noniodinated metabolite was facilitated by chemical structure of the corresponding iodinated metabolite. As a result, each noniodinated metabolite was detected based on the correct chemical formula provided by the accurate m/z, which was measured with a mass accuracy of <2 ppm (Supplemental Table 2).

Comparison of ICP-MS and ESI-MS Responses. The average ICP-MS and ESI-MS responses from five injections on-column of 0.25 pmol each of 127I-ACTH (4–10) and its four metabolite standards (M2–M4 and M6) are tabulated in Table 4. The CV for analyses by ICP-MS ranged from 6.0 to 11.3% compared with CV from 1.7 to 6.9% obtained by ESI-MS analyses. In general, there was +1.3- to twofold difference in the responses of metabolites of 127I-ACTH (4–10) by ICP-MS analysis compared with −4.5- to +1.5-fold difference in responses from ESI-MS analyses. There was greater deviation from equimolar responses of I-ACTH (4–10) and its metabolites by ESI-MS analysis than by ICP-MS analysis.

Discussion

Our initial attempt to couple capillary UPLC to an ICP-MS was not successful due to the rapid decline in signal sensitivity from plasma instability and leading eventually to extinction of the argon plasma during repeated analyses. The extinction of the argon plasma during ramping of the reverse-phase capillary LC gradient can be attributed to instability of the plasma from overloading with organic solvent vapor (Boorn and Browner, 1982; Stefánka et al., 2006; Pröfrock, 2010). Furthermore, this rapid decline in sensitivity was caused by deposition of carbon on the sampling and skimmer cones from incomplete decomposition of the organic molecules due to cooling of the argon plasma by the high organic solvent content (Blades and Caughlin, 1985; Sutton and Caruso, 1999; Wind et al., 2002; Stefánka et al., 2006; Pröfrock, 2010). We chose addition of a final concentration of 6% oxygen to the argon plasma (Takahata and Watanabe, 1993; Sutton and Caruso, 1999) to remove the carbon load together with reduction of spectral interference by collision with He in the CRC, and the application of kinetic energy discrimination led to the successful coupling of a reverse-phase capillary UPLC with ICP-MS for routine analysis of peptides.

However, it is not easy to predict the effect that oxygen-doped argon plasma has on the limit of detection (LOD) of elements by ICP-MS due to the oxide formation and spectral interferences (Durrant, 1993; Kralj and Veber, 2003; Amais et al., 2010). This capillary UPLC-ICP-MS method gave a LOD that is ~fourfold higher than the LOD of 122 fg 127I reported using reverse-phase capillary LC coupled to a single-stage quadrupole ICP-MS via an argon plasma (Pereira Navaza et al., 2009). This LOD cannot be attributed to dilution of the ion current by oxide formation as the formation of 127I2+ is thermodynamically unfavorable because it is an endothermic reaction (Chase, 1996). Instead, the different reported LOD is probably due to different instrumental setup and operation. It can be concluded that doping the argon plasma with small percentage of oxygen led to minimal decrease in sensitivity of analysis.

It has been reported that radiiodination can affect the biologic and/ or pharmacokinetic properties of a peptide (Loot et al., 2005; De Spiegeler et al., 2012). Hence, it is important to characterize the suitability of the bioactive radiiodinated peptide as a tracer for investigation of metabolism of the native peptide. This was the rationale for comparison of the metabolic stability of ACTH (4–10) and 127I-ACTH (4–10) in human liver S9, where the similar metabolic turnover of >80% for both peptides suggested that iodination at the para position of the phenylalanine residue of ACTH (4–10) had

| TABLE 4 |
| Comparison of ICP-MS and ESI-MS responses from on-column injection of 0.25 pmol each of 127I-ACTH (4–10) and its four metabolite standards (M2–M4 and M6) |

| Data are expressed as mean ± S.D. from five replicate analyses. |

<table>
<thead>
<tr>
<th>Capillary UPLC-ICP-MS</th>
<th>Capillary UPLC-ESI-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>127I-Phe</td>
<td>6141 ± 402</td>
</tr>
<tr>
<td>127I-ACTH (4–8)</td>
<td>22,182 ± 531</td>
</tr>
<tr>
<td>127I-ACTH (5–10)</td>
<td>42,475 ± 711</td>
</tr>
<tr>
<td>127I-ACTH (7–10)</td>
<td>7738 ± 537</td>
</tr>
<tr>
<td>127I-ACTH (4–10)</td>
<td>27,712 ± 1521</td>
</tr>
</tbody>
</table>
The inhibition of metabolism of $^{127}$I-ACTH (4–10) in liver S9 was mediated predominantly by cytosolic enzymes because the turnover in cytosol is similar to S9 and higher than in microsomes. Also, both liver microsomes and cytosol formed the same metabolites, except for the minor metabolite M1, which is only produced by liver cytosol. A comparison of turnover of $^{127}$I-ACTH (4–10) in various tissue preparations indicates that liver is the main organ for its metabolic clearance, with potential backup by kidney based on its ~fourfold lower turnover and with negligible contribution of plasma enzymes. Also, there is no involvement of cytochrome P450 enzymes because the metabolism was not NADPH-dependent. Instead, metabolism is mediated mainly by proteases based on the near-complete inhibition by addition of both a protease inhibitor cocktail and EDTA. There was inhibition of in vitro metabolism by AEBSF and bestatin in the cocktail, which is consistent with the involvement of serine peptidase and aminopeptidase, respectively. However, the failure of both AEBSF and bestatin to inhibit the metabolism of $^{127}$I-ACTH (4–10) in human liver S9 to the same extent as the protease inhibitor cocktail is consistent with a complex participation of multiple proteases. The lack of selective marker substrates and inhibitors, together with many peptidases in families and clans belonging to serine peptidase (Page and Di Cera, 2008) or aminopeptidase (Sanderink et al., 1988), created a challenge in the identification of the individual proteolytic enzyme responsible for the in vitro metabolism of $^{127}$I-ACTH (4–10), which is beyond the scope of this study.

Consistent with radioprofiling, this method has to rely on molecular mass spectrometry for elucidation of structures. The matching of retention times of metabolites relative to $^{127}$I-ACTH (4–10) provided by ICP-MS and LTQ/Orbitrap together with the chemical formulæ of precursor and product ions led to successful identification of $^{127}$I-tagged metabolites, as shown in Table 3. The confirmation of the proposed structures of certain metabolites was achieved by matching retention times with reference standards, as shown in Fig. 5, and also from comparison of product ion mass spectra (data not shown). The primary sequences of metabolites M4 to M6 are consistent with C-terminal peptide fragments because they retained the C-terminal glycine residue. Metabolites M4, M5, and M6 are postulated to derive from N-terminal cleavage of methionine, glutamic acid, and histidine residues from $^{127}$I-ACTH (4–10), M4, and M5, respectively. Likewise, M3 is the only N-terminal metabolite and postulated to form directly from C-terminal cleavage. The metabolites M3 and M6 are speculated to undergo proteolytic cleavage to M1, and further proteolysis of M1 led to the formation of the major M2 metabolite. The proposed N-terminal proteolytic cleavage pathway is supported by detection of M1, M2, M5, and M6 from incubation of M4 and also from detection of M1 and M2 from incubation of M6. Furthermore, this M2 was also the major metabolite produced from incubation of M3, M4, or M6 in human liver S9. All of these metabolites were also generated from incubation of native ACTH and provided evidence that tagging the para position of phenylalanine with $^{127}$I did not impact on the sites of cleavages. Also, there was no evidence for dehalogenation of the $^{127}$I-tag on phenylalanine of $^{127}$I-ACTH (4–10) and its six metabolites even though dehalogenation has been observed for iodinated tyrosine or histidine of obestatin peptides (De Spiegeleer et al., 2012). Taken together, these data suggest that the successful use of a $^{127}$I-tag as a metabolite tracer requires that the tag alters neither the metabolic stability nor site of proteolysis compared with the native peptide and also that the $^{127}$I-tag is not lost via metabolic dehalogenation.

The inhibition of metabolism of $^{127}$I-ACTH (4–10) in liver S9 by EDTA was not easy to detect because EDTA alone had negligible effect on the metabolic turnover of $^{127}$I-ACTH (4–10). However, near-complete inhibition of M5 formation was observed only when EDTA was added in combination with a protease inhibitor cocktail (Table 2). It is speculated that this cocktail unmasked the potential contribution of a metalloprotease to the metabolism of $^{127}$I-ACTH (4–10) to M5. The addition of the serine peptidase inhibitor (AEBSF) during incubation did not completely inhibit the formation of M2, but led to ~twofold increase and decrease in percentage of composition of M5 and M2, respectively, which revealed the potential involvement of a serine peptidase in an alternate secondary pathway for conversion of M5 to M2. In contrast, incubation with an aminopeptidase inhibitor (bestatin) led to ~90% decrease in M2 and is consistent with an aminopeptidase catalyzing the primary pathway to M2 formation. This inhibition of M2 formation was accompanied by ~2- and ~38-fold increase in percentage of composition contributed by M6 and M1, respectively, and resulted in M1 replacing M2 as one of the major in vitro metabolites. Therefore, it is speculated that bestatin inhibited the aminopeptidase responsible for the primary pathways for N-terminal cleavage of M6 to M1 and M2, and M1 to M2. These preliminary experiments with a limited number of inhibitors suggest a complex involvement of several proteolytic enzymes in the metabolism of $^{127}$I-ACTH (4–10), as illustrated in the proposed in vitro human metabolic pathway of $^{127}$I-ACTH (4–10) in Fig. 6.

Another desirable property of a metabolite tracer is to provide quantitative information on the formation of metabolites. ICP-MS gave closer approximation to equimolar responses of $^{127}$I-ACTH (4–10) and its metabolites compared with ESI-MS (Table 4) because ESI is prone to ion suppression (King et al., 2000). This led to the correct assignment of $^{127}$I-Phe (M2: ~51%) as the major in vitro metabolite in human S9 by ICP-MS compared with the identification of M2 (~25%), M4 (~26%), and M5 (~20%) as major in vitro metabolites by ESI-MS. The reason for this slight change in ICP-MS responses during reverse-phase gradient capillary UPLC analysis has already been reported (Kannamkumarath et al., 2004; Profröcke and Prange, 2009).

Thus, $^{127}$I has most of the desired attributes, but without the liability and safety issues of the radioactive $^{125}$I as a metabolite tracer. Other reasons for selecting $^{127}$I include leveraging extensive experiences of using $^{125}$I as a radiotracer for characterization of metabolism of peptide drugs (Vugmeyer et al., 2010), the ease in transferring $^{125}$I incorporation techniques for peptides to $^{127}$I (Schumacher and Tsonides, 1995), and the reasons for high sensitivity of $^{127}$I detection by ICP-MS (Gammelgaard et al., 2008).

Determining the in vitro–in vivo correlation of the metabolic data for preclinical species can help inform what might be the expected...
clearance pathways in humans in vivo following peptide disposition studies in human tissue preparations in vitro. In addition, the application of this tool to map the relative contribution of liver, kidney, and other peripheral tissues to peptide drug clearance together with the understanding of the contribution of biliary excretion and glomerular filtration to its elimination will be helpful in predicting the impact of hepatic or renal impairment on its pharmacokinetics. Such data may be used to position the peptide drug relative to heptatically or renally impaired special populations of patients. For example, iraglutide is more suitable than exenatide in management of type 2 diabetes in patients with severe renal impairment due to reduced clearance of exenatide (Linnebjerg et al., 2007), but not iraglutide (Jacobson et al., 2009), in this special population of patients. This is because exenatide is cleared primarily by renal elimination (Simonsen et al., 2006) compared with iraglutide, which is extensively metabolized by proteolysis in the liver and other peripheral tissues (Malm-Erjefält et al., 2010).

In summary, this metabolite tracer technique is a useful addition to the drug metabolism and pharmacokinetics (DMPK) toolbox for simultaneous investigation of metabolic turnover and profiling of 121I-tagged peptide therapeutics using ICP-MS. Any peptide fragment retaining the 121I-tag will be detected as a peak in the 121I-RIC. The potential for equimolar responses of 121I using ICP-MS provided convincing arguments for this nonradioactive approach to supplant radioiodination to investigate the in vitro metabolic fate of a peptide therapeutic.

Acknowledgments

The authors thank Dr. Amir Liba (Agilent Technologies, Wilmington, DE) for consultation on hyphenation of capillary UPLC to ICP-MS and operation of ICP-MS.

Authorship Contributions

Participated in research design: Lim, Cao, Qiu, Silva.
Conducted experiments: Cao, Lim, Qiu.
Contributed new reagents or analytic tools: Cao, Lim.
Performed data analysis: Lim, Cao.
Wrote or contributed to the writing of the manuscript: Lim, Evans.

References