Correspondence case report

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Synthesis and characterization of the N-terminal acetylated 17-23 fragment of thymosin beta 4 identified in TB-500, a product suspected to possess doping potential

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Introduction

According to the Prohibited List of the World Anti-Doping Agency (WADA), the use of any unapproved drug for human therapeutic use is strictly prohibited in sports.[1]

The use of performance-enhancing peptides is a growing issue in human sports drug testing[2–5] as well as in equine sports drug testing.[6]

A product called TB-500, claimed to increase muscle growth and tissue repair in horses and other mammals, is available on the Internet and officially distributed. It is presented as 'the synthetic peptide of the active region of thymosin beta 4 (Tβ4)', without any further qualitative description such as amino acid sequence or molecular weight. Tβ4, a 43 amino acid peptide, is the most abundant member of beta thymosins.[7] Its primary function is to stimulate the production of T cells, which are an important part of the immune system. Additionally, Tβ4 has been shown to prevent apoptosis, promote cell survival, angiogenesis, and tissue regeneration in mice and rats.[8]

TB-500 is intended for veterinary use only (10 mg/dose), but it is legitimate to suspect its use as doping agent not only in horse and greyhound racing, but even in humans. Indeed, TB-500 packages, containing six powder vials and physiological saline solutions for injection, have already been confiscated by legal officials. In at least one case this received worldwide attention from the media since it was connected to a former professional athlete which was still employed by his team,[9] who declared that the product was for personal use. In another case, a former team doctor was arrested in possession of the substance.[10] These events suggest that TB-500 might also be used by athletes for doping purposes, although no evidence of misuse has been reported so far.

A preventive and proactive approach against these novel formulations containing peptidic drugs without any clinical approval is mandatory, including identification of the active content of these products, in order to monitor their misuse. Additionally, methods need to be developed for their detection and preferably, well characterized reference standards need to be synthetized. The aim of this work was therefore to identify, to synthesize and suggest an analytical strategy to detect the active compound in plasma and urine.

Materials and methods

Chemicals and reagents

All reagents were analytical or high performance liquid chromatography (HPLC) grade. Reference standard of human Tβ4 was purchased from Bachem (Weil am Rhein, Germany).

Acetonitrile, water, dichloromethane were purchased from BioSolve (Lexington, MA, USA). Glacial acetic acid was purchased from Merck (Darmstadt, Germany); Methanol, formic acid, N,N'-diisopropylcarbodiimide (DIC), 4-dimethylaminopyridine (DMAP), N,N-diisopropylethylamine (DIPEA), piperidine, pyridine, (methyl-t-butyl ether (MTBE), and acetic anhydride were purchased from Sigma-Aldrich (St Louis, MO, USA).

All the fluorenylmethyloxycarbonyl chloride (Fmoc) protected amino acids were L-isomers. Fmoc-Lys(Boc)OH, Fmoc-Thr(tBu)OH, Fmoc-Glu(tBu)OH were purchased from Nova Biochem (San Diego, CA, USA). Fmoc-Leu-OH precoated WANG-Resin was purchased from Bachem. Fmoc-Gln(Trt)OH and O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) from Iris Biotech GnBH (Marktredwitz, Germany).

TB-500

A TB-500 package, confiscated by Belgian Customs during a routine control, was delivered to the laboratory. The sealed vial contained approximately 10 mg of a white powder. Stock (1 mg/ml) and working solutions (100 μg/ml and 100 ng/ml) of TB-500 and human Tβ4 from lyophilized powders were prepared by dissolving them in aqueous acetic acid (2% *v/v*).

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Liquid chromatography

Chromatographic separation was achieved with a Surveyor MS Pump Plus coupled with a Surveyor Plus autosampler (Thermo Scientific, Bremen, Germany) using a Zorbax RX-C8 reverse-phase column (2.1 x 150 mm, 5 μm) from Agilent Technologies (Santa Clara, CA, USA). The conditions were the same for both high-resolution and low-resolution MS experiments. For each sample, 5 μl were injected. A binary gradient was used: mobile phase A consisted of water containing 0.2% formic acid; mobile phase B consisted of acetonitrile containing 0.2% formic acid. Gradient elution was as follows: it started from 100% A for 5 min, then decreased linearly to 0% A in 10 min, and held at 0% A for 2 min. Finally, 95% A was eluted for 3 min to equilibrate the system for further injection experiments, for a total run time of 20 min. A constant flow rate of 300 μl/min was maintained.

Mass spectrometry

High-resolution mass spectrometry (HRMS) experiments were performed on an Exactive benchtop, Orbitrap-based mass spectrometer (Thermo Scientific, Bremen, Germany). The instrument operated both in positive full-scan MS mode and positive higher-energy collision dissociation (HCD) 'all ion fragmentation' MS/MS mode at 25 eV. The sheath gas (nitrogen) pressure was set to 60 (arbitrary units), the auxiliary gas (nitrogen) pressure set to 30 (arbitrary units) and the capillary temperature set to 350°C. The capillary voltage and spray voltage were set to 30 V and 3 kV, respectively. The instrument operated in full scan mode from *m/z* 100–2000 at 100 000 resolving power. The data acquisition rate was 1 Hz. Approximately 10 scans were averaged per spectrum.

Low-resolution MS/MS characterization of TB-500 was performed on a TSQ Quantum Discovery Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with an ESI source operating in positive mode The ESI–MS operating variables used in this study were as follows: capillary voltage, 3.5 kV; source temperature, 350°C; sheath gas (nitrogen) pressure, 30 psi; auxiliary gas (nitrogen) pressure, 10 psi; tube lens offset, 84 V.

Data analysis

ProMass software from Thermo Fisher (Bremen, Germany) was used for spectral deconvolution. Protein Prospector v 5.8.0 (http://prospector.ucsf.edu/prospector) was used to compare and match acquired HCD spectra to those predicted *in silico*. Mass tolerance was set at 5 ppm.

Synthesis of the peptide

Ac-Tβ4(17-23) was synthesized using a standard Fmoc-SPS protocol.[11] The C-terminal amino acid (FmocGln(Trt)OH) was already coupled to WANG-resin with 0.63 mmol/g loading. Subsequent coupling of amino acids was achieved using 3 eq amino acid, 3 eq HBTU as activator and 6 eq DIPEA as base in DMF. After each

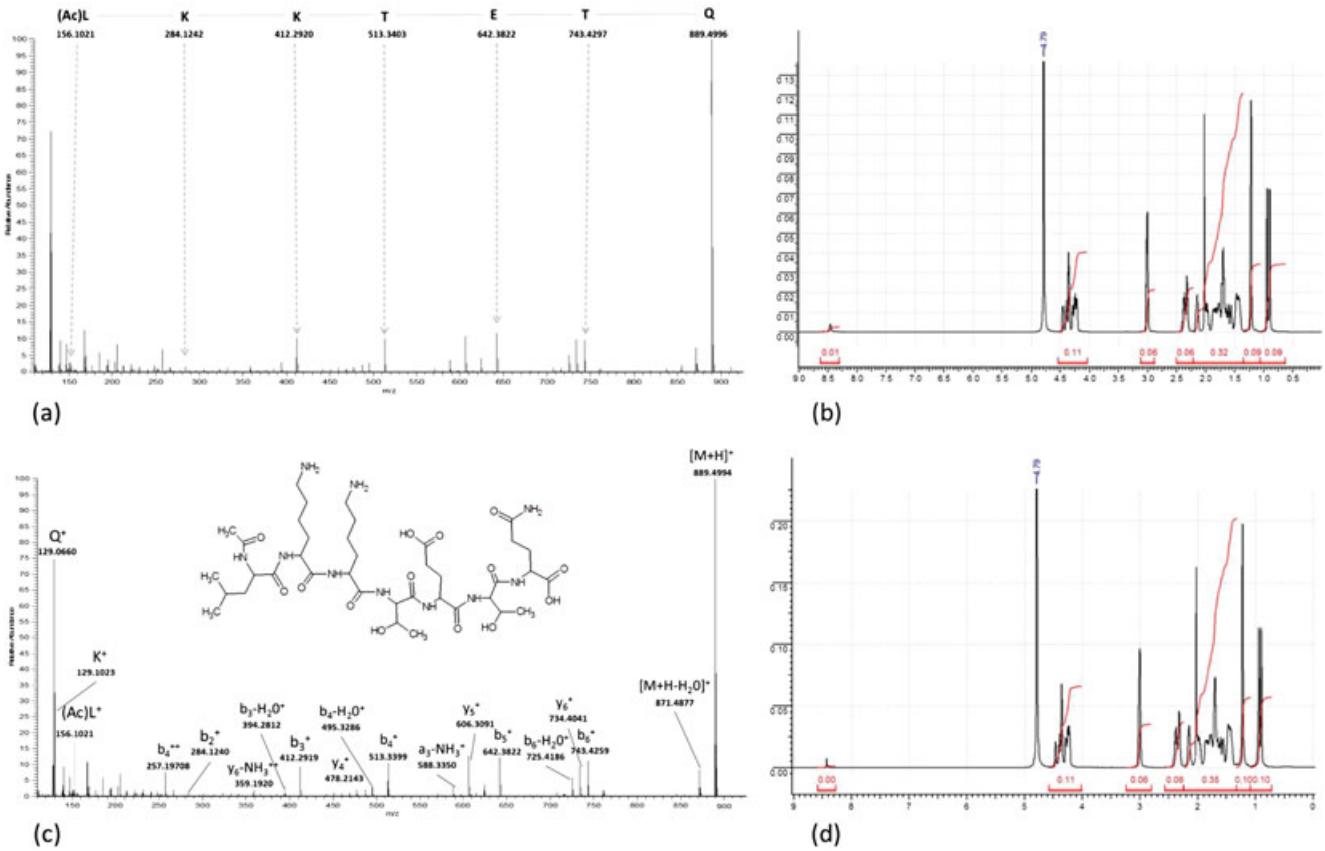

Figure 1. HCD MS/MS recorded at 20 eV (a) and 700 MHz-NMR (b) spectra of TB-500. The structure of the peptide was confirmed after synthesis of the Ac-Tβ4(17-23), which presents identical MS/MS (c) and NMR (d) spectra.

coupling, including the coupling to WANG-resin the protective Fmoc group was removed with 20% (*v/v*) piperidine in DMF. Acetylation of the N-terminus was achieved with 1M acetic anhydride in pyridine. The peptide was cleaved from the resin using Reagent K[12] and precipitated in MTBE.

Analysis of spiked plasma and urine

Two ml of blank plasma and 2 ml of blank urine were spiked with the peptide at 500 pg/ml. Two ml of saturated ammonium sulfate (Merck, Darmstadt, Germany) solution were added to plasma and the samples were centrifuged (4100 g, 1 h, 2°C) to precipitate plasma proteins. Both plasma and urine were then acidified with 50 µl of 10% formic acid and processed by solid-phase extraction (SPE) using Oasis MCX (60 mg) cartridges, purchased from Waters (Milford, PA, USA). The column was first activated with 2 ml of methanol and subsequently rinsed with 2 ml of 5% acetic acid. After the sample was loaded, the column was washed first with 2 ml of 5% acetic acid, then with 2 ml of methanol. Finally, samples were eluted with 1.25 ml of a solution consisting of 80:20 methanol:5% ammonia. The eluate was evaporated to dryness with a centrifugal evaporator (45°C, 240 g), and then dissolved in 100 µl of 95:5 water:acetonitrile, containing 0.1% formic acid.

Results

HRMS identification of the active content of TB-500

Solutions of TB-500 were first injected for HPLC-HRMS analysis in order to identify the active ingredient(s) in the formulation. Total ion chromatogram showed one main peak, which could be assigned to originate from a peptide-like structure at $t_r = 9.1$ min. The full-scan MS spectrum (data not shown) revealed the presence of two molecular ions of the same molecule, respectively 445.2532 ($[M+2H]^{2+}$) and 889.4988 ($[M+H]^{+}$). Spectrum deconvolution provided a molecular weight (MW) of 888.4910. It was immediately evident that TB-500 did not contain the endogenous Tβ4 (MW: 4963.4642). Therefore, elucidation of the structure of the detected species was accomplished by *de novo* sequencing based on HCD of the peptide precursor at 25 eV. b^+ and y^+ ions allowed *de novo* sequencing of the peptide through direct matching with theoretical data. As shown in Figure 1a and summarized in Table 1, a heptapeptide was detected, corresponding to the 17–23 fragment of human Tβ4 (sequence: LKKTETQ). Additionally, all b^+ ions showed a positive *m/z* shift of 42.0105 with respect to the Tβ4 fragment, indicating the presence of an acetylated N-terminus. The identified peptide (theoretical MW 888.4911) will be further referred to as Ac-Tβ4(17-23). Finally, a confirmation of the structure was given after synthesis of the peptide, as shown by the HCD spectrum depicted in Figure 1c, by comparison of respective NMR spectra in Figure 1b and 1d, and the chromatograms in Figure 2. When a gradient with a slower increase was used for TB-500 (0% to 20% B in 10 min instead of 0% to 100% B) to check for the presence of coeluting substances or stereoisomeric impurities still a single peak was observed (data not shown). No other active substances or excipients were detected during the analysis.

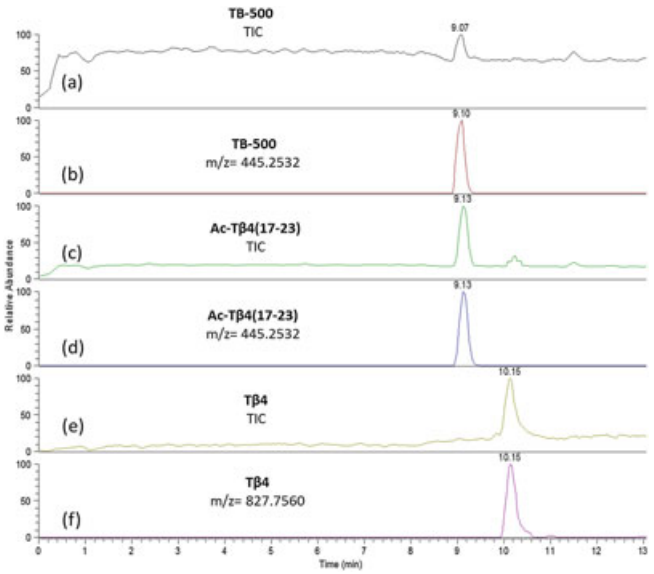

Figure 2. TIC and base peak chromatogram for TB-500 (a, b), the synthesized Ac-Tβ4(17-23) (c, d), and Tβ4 (e, f).

Low-resolution MS/MS of Ac-Tβ4(17-23)

Low resolution MS/MS data can provide useful information to monitor in a sensitive way the presence of these substances by selected reaction monitoring (SRM).

The product ion scan spectrum (20 eV) of Ac-Tβ4(17-23) of the parent ion $[M+2H]^{2+}$ at *m/z* 445.5 shows an elevated number of fragments, including the complete *y* and *b* ion series and several internal fragments. These results are depicted in Figure 3. Additionally, Table 2 shows the results from optimization of MS parameters for the SRM detection of the compound. The product ions at *m/z* 84.1 and 129.1, related to the glutamine and lysine residues were the most abundant.

Table 1. Identification of Ac-Tβ4(17-23) based on matching between the experimental and *in silico* data on *b* ions from HCD experiments

$(b)^+_{theor}$	$(b)^+_{exp}$	Δppm		Amino acid		$(y)^+_{theor}$	$(y)^+_{exp}$	Δppm
156.1019	156.1020	−0.6	1	(Acetyl)L	7	-	-	-
284.1969	284.1969	0.0	2	K	6	734.4043	734.4042	0.1
412.2918	412.2918	0.0	3	K	5	606.3093	606.3094	−0.2
513.3395	513.3396	−0.2	4	T	4	478.2144	478.2145	−0.2
642.3821	642.3817	0.6	5	E	3	377.1667	377.1670	−0.8
743.4298	743.4297	0.1	6	T	2	248.1241	248.1240	0.4
-	-	-	7	Q	1	147.0764	147.0765	−0.7

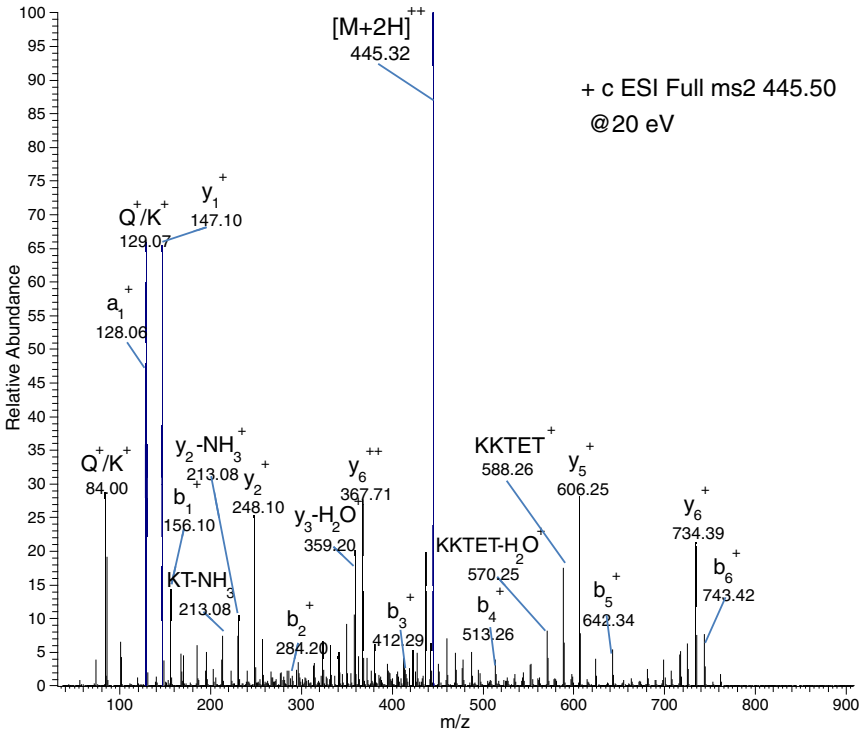

Figure 3. Full scan MSMS spectrum of Ac-Tβ4(17-23) at 20 eV.

Table 2. Main results from MSMS optimization of Ac-Tβ4(17-23) from TB-500

Precursorion (m/z)	Production (m/z)	Fragment	Collision E (ev)	Abundance (%)
445.5	84.1	Q/K	41	100
	129.1	Q/K	28	92
	86.1	L	34	55
	606.4	y_5	17	16
	588.3	KKTET	18	9

Discussion

TB-500 is one of several formulations available on the online market that contain a peptidic drug which is claimed to enhance sport performances. Although it is marketed only for veterinary use, it is sold to non-veterinarians as well via the Internet and there is anecdotal evidence that is probably used by athletes. In recent years, several research groups have investigated and highlighted the increasing problem of the use of non-approved designer peptides,[2–5] which were often only identified after the products had been confiscated by Customs Police officers.

Ac-Tβ4(17-23) corresponds to the acetyl derivative of the major actin-binding site of Tβ4, that has been shown to promote angiogenesis, wound healing and hair growth *in vitro* and in animal models.[13] Therefore, the plausible rationale for use of TB-500 in doping lies in the role of Ac-Tβ4(17–23) in tissue growth and regeneration, which can be particularly important for recovery after intense workout or competition. The non-acetylated form of the peptide has been detected *in vivo* as an endogenous degradation product of Tβ4,[13] whereas the acetylated form is not described. N-terminal acetylation is a common strategy in drug development of peptides to increase the half-life, and therefore the activity, of peptides.[14] This acetylation should allow for a clear differentiation between endogenous and exogenous Tβ4 in biological samples, although metabolic deacetylation should be investigated. The absence of reference standards for most of the black market peptides, including Ac-Tβ4 (17-23), presents a problem in doping control concerning the fulfillment of compound identification criteria. Moreover, some of these products contain different ingredients as officially claimed in the product description These differences include amino acid substitution, truncation, or other chemical modifications. Additionally, due to the lack of information on pharmacokinetic properties of this peptide and the ethical impossibility to administer these unknown preparations to healthy volunteers, its urinary or serum detection can generally only be postulated. Due to its relatively low molecular weight, renal excretion could be expected, with sub-nanogram urinary concentration similarly to other oligopeptides[15,16] but this educated guess needs to be confirmed experimentally. However, in order to provide useful indication for the detection of the intact peptide, urine samples were spiked with Ac-Tβ4(17-23) and processed by protein precipitation (only for plasma samples) and solid-phase extraction (SPE) before LC-MS analysis. Figure 4 shows the detection of Ac-Tβ4(17-23) both in urine and plasma spiked at a concentration of 0.5 ng, using product ions at *m/z* 129.0 and 606.4 from precursor ion at *m/z* 445.5. Awaiting more pharmacokinetical information from approved administration studies to humans or, taking into account the ethical aspects more likely, other species, the authors suggest the monitoring of misuse of this peptide in plasma and/or urine with these protocols.

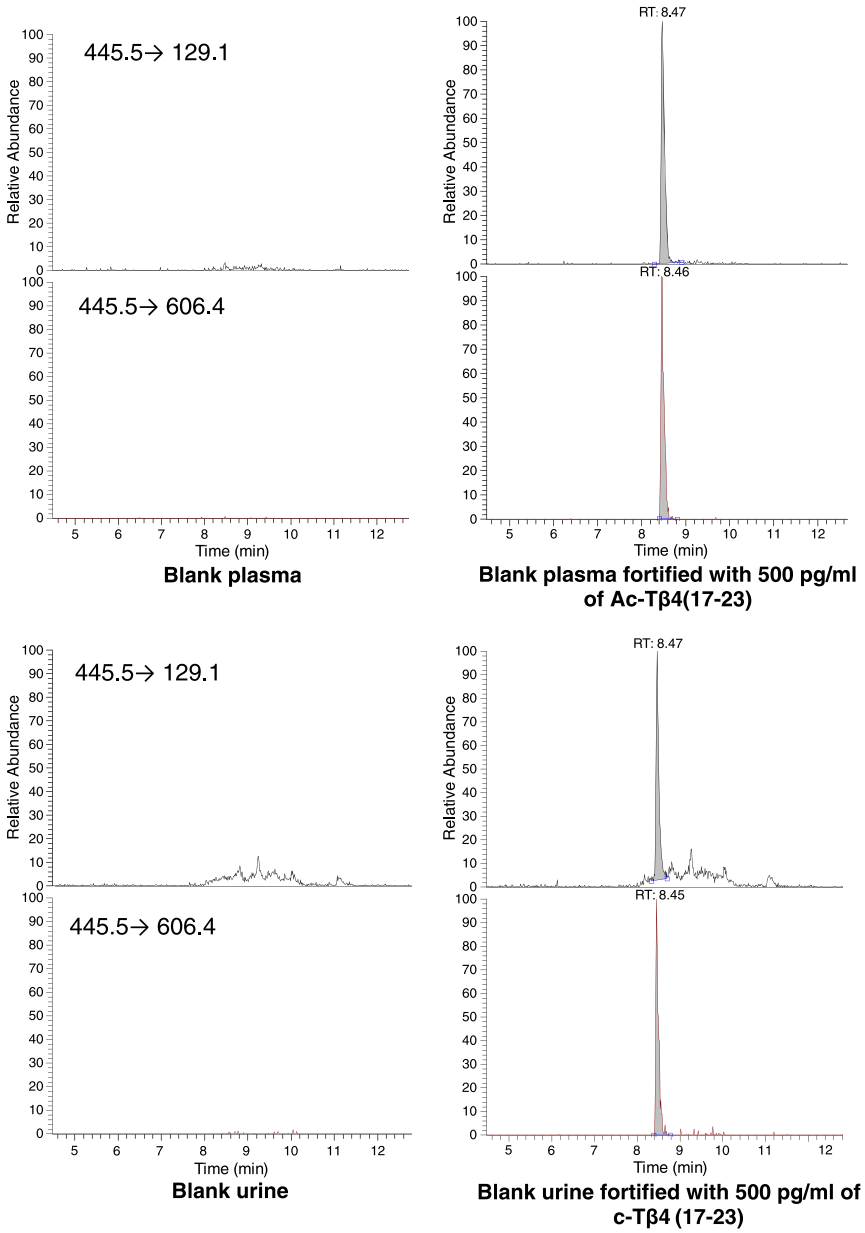

Figure 4. Detection of Ac-Tβ4(17-23) in plasma (b) and urine (d) after fortification with 0.5 ng of peptide. The peptide was not detected in blank plasma (a) or in blank urine (c).

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