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Identification and determination of degradation products of human insulin and its long acting synthetic analogues in urine by means of ESI-LC-MS/MS

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Introduction

The potential performance enhancing effects of insulin in elite sports is frequently discussed and reported with particularly life threatening consequences for the cheating athletes (1-5). The pharmaceutical market provided different formulations of recombinat human insulin as well as various rapid-, intermediate- and long acting synthetic analogues to ensure comprehensive treatment of *diabetes mellitus* (6, 7).

After successful implementation of an analytical method to determine the potential misuse of rapid acting insulin analogues in regular urinary doping control samples, this study should ensure the elucidation of sufficient target analytes to uncover an application of long acting synthetic insulins as Lantus (LAN, Glargine) and Levemir (LEV, Detemir) in urine samples of cheating athletes (8, 9). LAN is a synthetic insulin that differs in its amino acid sequence from human insulin (HI) by exchanging the amino acid residue asparagine to glycine at position 21 of the A-chain, and the B-chain is prolonged by two additional arginine residues at position B31 and B32 (Fig. 1) (9). These modifications realize microprecitation, due to an elevated isoelectric point of the active substance, in the subcutaneous tissue at physiological pH values and, thus, an effectively prolonged time of onset (6). LEV (Lys^{B29}-(N^{ε}-tetradecanoyl)- Des(B30) human insulin is an other insulin analogue with protracted bioavailability, posseses a combined increase of self association to hexameric aggregates and albumin binding due to acylation of the amino acid residue B29-lysine with myristic acid (Fig. 1). The bioactivity of this substance is approximately four fold decreased compared to HI (6).

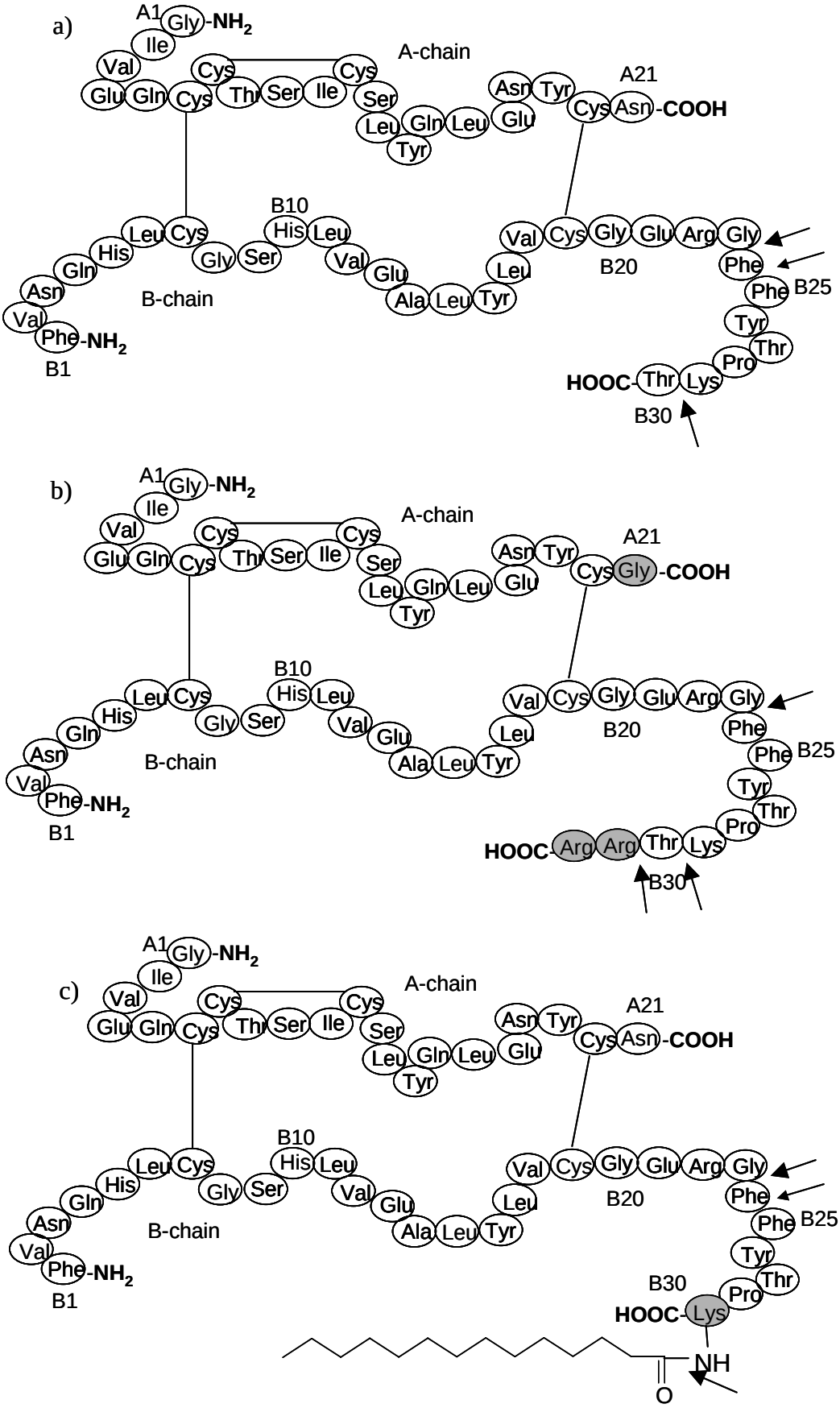

Fig. 1: Primary structures of a) Human Insulin, b) Lantus and c) Levemir. Modifications are market grey and identified cleavage sites are shown with arrows.

The delayed release of the intermediate acting recombinant human insulin formulations (e.g. NPH-insulins) is attributable to the addition of protamine that leads to reversible protamine binding after application in the subcutaneous tissue (10).

In contrast to earlier results, investigations with urine samples obtained from diabetic patients treated with LAN or LEV resulted in no analytical findings of the intact substances in those samples.

Thus, further investigations focused on partly degraded metabolic products of insulin occuring in urine (8, 11). Well known metabolic degradation processes are mainly catalysed by the endogenous enzymes Insulin-Degrading-Enzyme (IDE) and Endosomal-Acidic-Insulinase (EAI), and first experiments started more than 30 years ago (12, 13). In these studies several cleavage sites at positions A13/14, A14/15, B9/10, B13/14, B16/17, B24/25 und B25/26 were identified utilizing *in-vitro* incubation of ^{125}I-labeled insulin with hepatocytes and subsequent separation by means of liquid-chromatography (12, 14-16). In the present study, new cleavage sites of HI, LAN and LEV were identified by LC-MS/MS determination of the respective degradation products in urine samples (Fig. 1).

Experimental

Materials and chemicals. Acetonitrile, trisodium phosphate dodecahydrate (p.a.), sodium chloride (p.a.) and acetic acid (glacial) were purchased from Merck (Darmstadt, Germany). OASIS HLB solid-phase extraction cartridges were obtained from Waters (Milford, MA). Trifluoracetic acid (99+%), tris(carboxyethyl)phosphine hydrochloride (TCEP-HCl), endoproteinase Lys-C from *Lysobacter enzymogenes*, and bovine insulin were from Sigma (St. Louis, MO). Lantus (Insulin Glargine), Levemir (Insulin Detemir), and recombinant human insulin were supplied by Novo Nordisk (Princeton, NJ), Aventis (Kansas City, MO), and Aventis (Frankfurt, Germany), respectively. DesB30 human insulin was kindly provided by Dr. T. Hoeg-Jensen from Novo Nordisk (Baegsvaerd, Denmark). The anti-insulin immunoaffinity-gel (0.5 mL/IAC, 10 mg IgG/mL) was obtained from CER (Marloie, Belgium).

Urine samples. For all experiments and validation steps urine samples from healthy male and female volunteers were utilized. In addition, urine samples from athletes and patients, suffering from *diabetes mellitus* and declaring the continual regimen with exogenous insulin, were analyzed.

Hydrolysis of LAN. Enzymatic hydrolysis of LAN was performed using endoproteinase Lys-C from *Lysobacter enzymogenes* to obtain DesB30-32 LAN reference compound. To 1 mL of

a solution containing 10 pmol/µL of LAN in 100 mM ammonium bicarbonate (pH 7.5) was added 20 µL of reconstituted enzyme solution (4.8 units/mL), and the mixture was incubated at 37 °C for 2 h. The hydrolysis was stopped by adding 20 µL of glacial acetic acid.

Stock and working solutions. Ten pmol/µL of bovine insulin in acetic acid (2 %) was used as internal standard stock solution. DesB30 HI and DesB30-32 LAN stock solutions contained 10 pmol/µL in acetic acid (2%) and were freshly diluted before use to a final concentration of 0.01 pmol/µL in acetic acid (2%). These working standard solutions also contained a ten fold carrier-excess of bovine insulin (0.1 pmol/µL), which was added prior to target analytes in order to saturate active surfaces of containers. All dilution steps were performed in polypropylene tubes, and stock solutions, stored at 2-8 °C, were found to be stable for one month.

Mass spectrometry. Mass spectra of reference compounds and urine samples were performed on an Applied Biosystems Qtrap 4000 mass spectrometer (Foster City, CA) using an electrospray ion source in positive mode. Selected mass spectrometric parameters are summarized in Table 1.

Table 1: Selected mass spectrometric parameters and validation results.

analytes	MW	precursor	coll. offset	recovery	LOD (*S/N>3*)	precision	cal curve (25 - 200 pg/mL)				stability
	[Da]	ion *m/z*	voltage [V]	[%]	[pg/mL]	at LOD [%]	R	intercept	slope	approx.	≤4 °C
DesB30 HI	5706.6	1142.3	75	109	25	12	0.988	0.242	0.006	linear	8 weeks
DesB24-30 HI	4922.6	1231.7	75	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
DesB25-30 HI	5069.8	1268.5	75	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
DesB30-32 LAN	5649.5	1130.9	75	97	25	14	0.995	0.075	0.024	linear	8 weeks
DesB31-32 LAN	5750.6	1151.1	75	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
DesB24-32 LAN	4865.6	1217.4	75	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Additionally, identity and purity of the utilized reference compounds DesB30 HI and DesB30-32 LAN were proven by measurements using a 1100 Series capillary liquid chromatograph from Agilent (Palo Alto, CA) coupled to an LTQ-Orbitrap mass spectrometer from Thermo (Bremen, Germany). Deconvolution of ESI-full scan spectra confirmed the accurate molecular masses of 5706.6 for DesB 30 HI and 5649.5 for DesB30-32 LAN.

Sample Preparation. The sample preparation procedure was adapted from a formerly developed method and is described in detail, elsewhere (8). Briefly, 15-25 mL of urine was fortified with internal standard, acidified, and vortex mixed. After OASIS solid-phase extraction (SPE), the cartridge was eluted directly onto the IAC. The IAC-gel sample mixture was incubated for 30 min at room temperature, and target analytes were eluted onto another

SPE-cartridge. Concentrated extracts were evaporated to dryness, reconstituted and analyzed by LC-MS/MS.

LC-MS/MS. LC was performed on an Agilent 1100 Series high performance liquid chromatograph (Palo Alto, CA) coupled to an Applied Biosystems Qtrap 4000 mass spectrometer (Foster City, CA). The LC was equipped with a Zorbax StableBond guard column (1 mm x 17 mm, 5 µm paticle size) and a Zorbax 300SB-C18 analytical column (1 mm x 50 mm, 5 µm particle size, 300 Å pore size) with an abient column oven temperature of 40 °C. Mobile phases consisted of 0.1 % of acetic acid with 0.01 % of TFA (phase A) and a mixture of 0.1 % acetic acid with 0.01 % TFA and acetonitrile (2:8, v:v) (phase B). The gradient started at 72 % A, ending at 35 % A after 15 min with a flow of 70 µL/min. Subsequently a 25 min equilibration time was added. The mass spectrometer operated in positive ion spray mode with a needle voltage of 5500 V. Parameters such as declustering potentials, iontrap filltime, and entry barrier voltage were optimised for isolation and detection of the fivefold protonated molecules of HI, DesB30 HI, DesB30-32 LAN and DesB31-32 LAN. Product ion spectra were measured at collision energies of 75 eV utilizing nitrogen as collision gas (6×10^{-3} Pa).

Validation Parameter. All validation steps for qualitative determination were affected by the lack of available reference compounds and, thus were focused on DesB30 HI and DesB30-32 LAN, only. Specificity was shown by preparing 10 different urine samples as described above. The limit of detection (LOD) was determined by comparison of the signal-to-noise ratios in blank- and fortified (25 pg/mL) samples. Additionally, the relative standard deviations for a sixfold determination at the LOD were calculated. The recovery of target analytes were defined by analysis and comparison of twelve (n = 6+6) urine samples, fortified (200 pg/mL) prior to and after sample preparation. Another set of samples was spiked with 25, 50, 75, 100, 125, 150 and 200 pg/mL of each reference compound and measured once to demonstrate the linearity of the signal ratios in this concentration range. The stability of the analytes in urine was proven by analyses of fortified sample aliquots before and after 2, 4 and 8 weeks storage time at 4 resp. –20 °C.

Results and Discussion

Identification of Degradation Products. Due to the fact that in administration study urine samples from patients treated with long acting analogues, intact insulins were barely detectable, the search for metabolic degradation products occuring in the respective urine samples were initiated. First investigations focus on already known degradation products, that

were identified by *in-vitro* experiments after exposition of ^{125}I-labelled insulin to enzymatic activities using various cell cultures in former studies (12, 14-16). Unfortunately, with the exemption of DesB25-30 HI, that was found in minor amounts, none of these metabolites were recovered in urine samples. But further investigations considering truncated B-chains allowed the mass spectrometric identification of to date unknown degradation products of HI, LEV and LAN. In an excretion study urine obtained from a patient treated with LEV, the metabolites DesB30 HI and DesB24-30 HI were determined, in addition to minor amounts of DesB25-30 HI.

Mass spectrometric identification of degradation products of HI and LEV were performed using product ion experiments of the five- resp. four-fold protonated precursor ions $[M+5H]^{5+}$, $[M+4H]^{4+}$ at *m/z* 1142 for DesB30 HI, *m/z* 1231 for DesB24-30 HI and *m/z* 1268 for DesB25-30 HI with abundant signals at 17.5, 16.4 and 17.1 min (Fig. 2) . The corresponding product ion spectra enable the identification of the compounds by characteristic product ion fragments mainly deriving from the C-terminus of the B-chain.

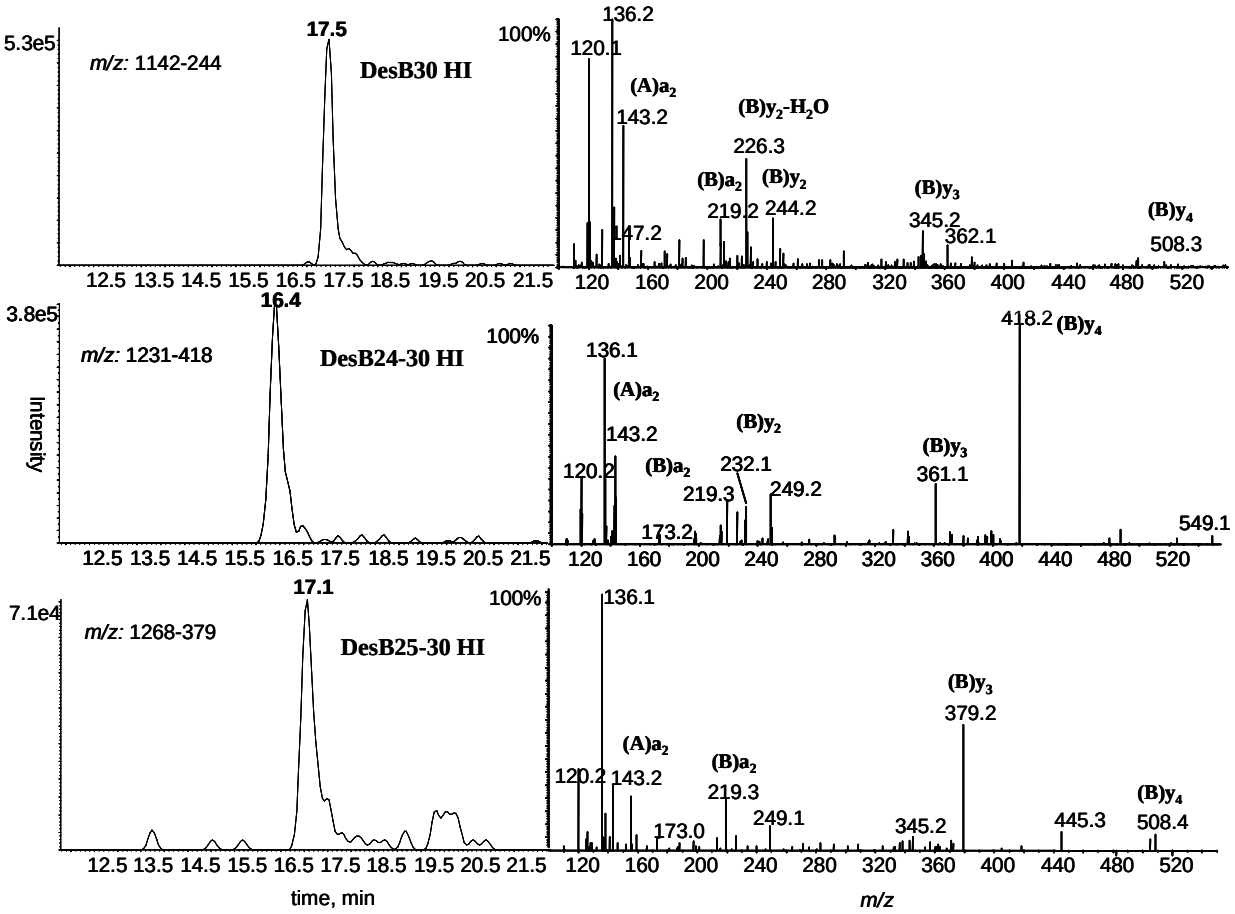

Fig. 2: Extracted product ion chromatograms with corresponding MS/MS-spectra of the five resp. fourfold protonated molecules of DesB30 HI, DesB24-30 HI and DesB25-30 HI at *m/z* 1142, 1231 resp. 1268 with abundant signals at 17.5, 16.4 and 17.1 min.

Additionally, the unambiguous confirmation of the results were approved by analysis of the cleaved B-chains after reduction with TCEP-HCl. The product ion chromatogram with respective product ion spectra of DesB24-30 HI, acquired from a urine sample from an athlete treated with long acting recombinant HI, is shown in Fig. 3.

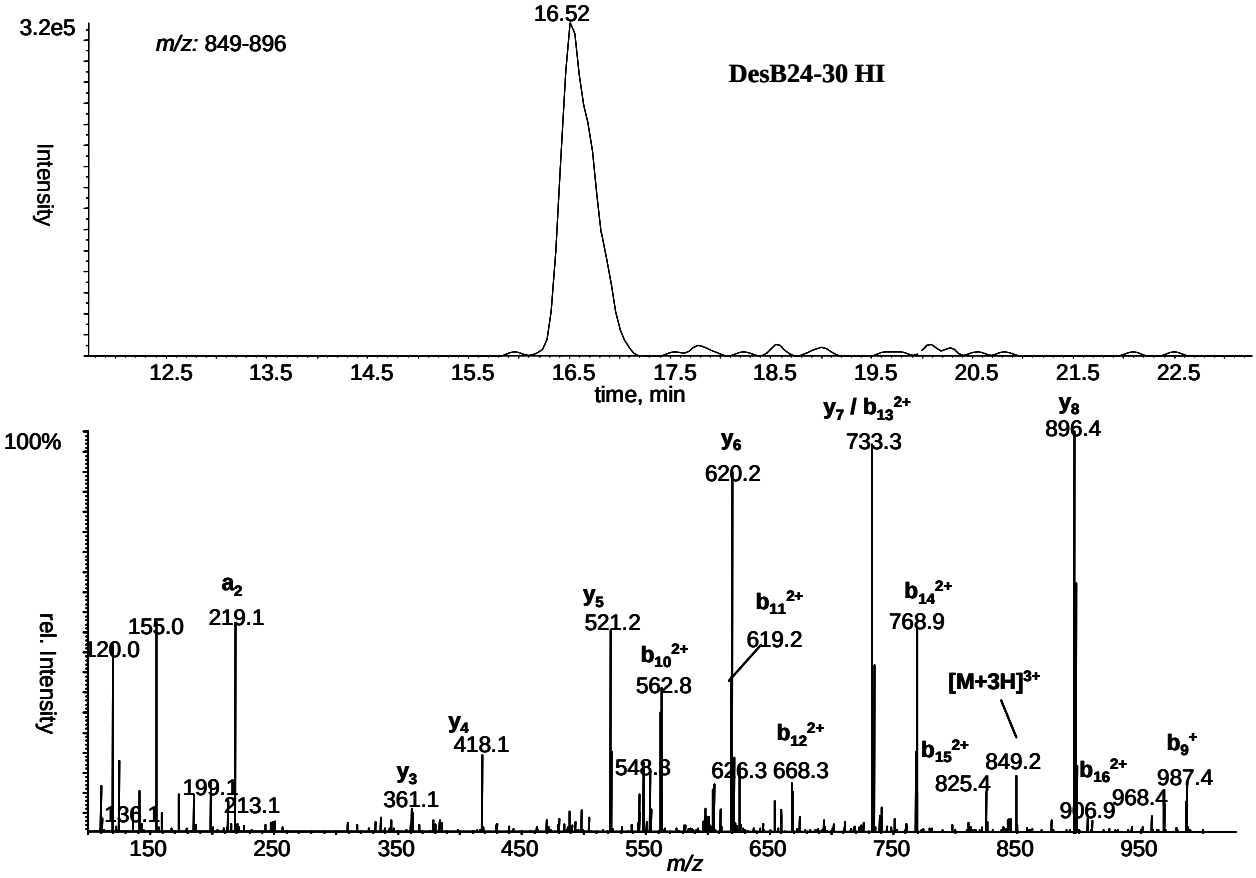

Fig. 3: Extracted product ion chromatogram with corresponding MS/MS-spectrum of the threefold protonated molecule of DesB24-30 HI-Bchain at *m/z* 849.2 with an abundant signal at 16.5 min and comprehensive amino acid sequence information of diagnostic y- and b-ions.

The threefold protonated precursor $[M+3H]^{3+}$ at *m/z* 849 of DesB24-30 HI-B-chain yielded a product ion spectrum providing a comprehensive amino acid sequence information comprising a continuously series of doubly charged b-ions and singly charged y-ions.

In almost the same manner the degradation products of LAN were identified by using the five- resp. fourth-fold protonated precursor ions $[M+5H]^{5+}$ resp. $[M+4H]^{4+}$ at *m/z* 1131 for DesB30-32 LAN, *m/z* 1151 for DesB31-32 LAN and *m/z* 1217 for DesB24-32 LAN with abundant signals at 17.2, 16.8 and 16.3 min (Fig. 4). Diagnostic product ions mainly deriving from the C-terminus of the B-chain enabled the identification and, the persistent A-chain modification ensured the differentiation from HI degradation products.

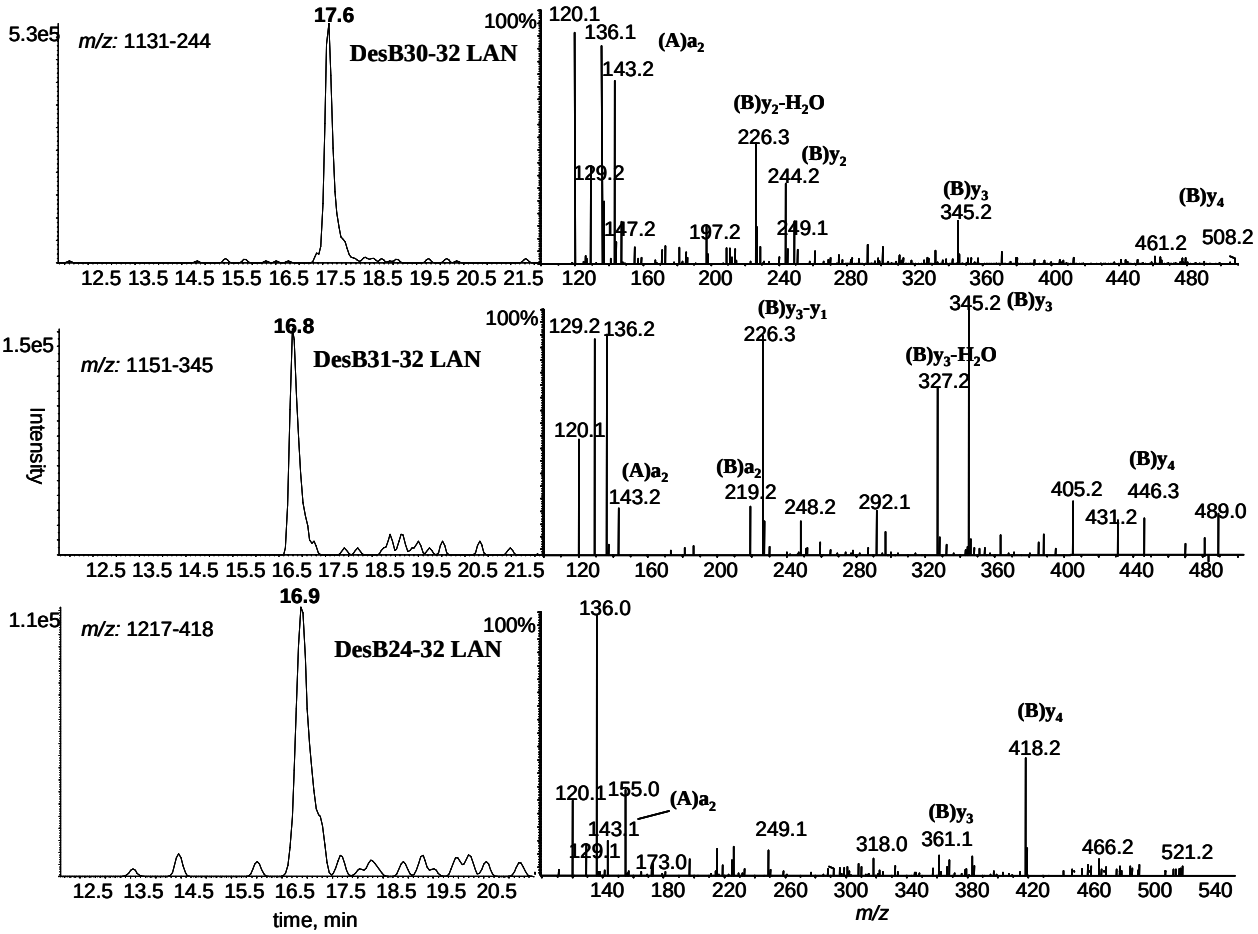

Fig. 4: Extracted product ion chromatograms with corresponding MS/MS-spectra of the five resp. fourfold protonated molecules of DesB30-32 LAN, DesB31-32 LAN and DesB24-32 LAN at *m/z* 1131, 1151 resp. 1217 with abundant signals at 17.6, 16.8 and 16.9 min.

Doping control aspects. The occurrence and detection of the identified degradation products of LAN DesB30-32-, DesB31-32- and DesB24-32 LAN enable the direct evidence of a LAN application due the persistent modification at position A21 of the amino acid sequence of the A-chain and, thus, an unambiguous distinction from endogenous origin. Unfortunately, such a direct evidence for a surreptitious LEV or recombinant HI application is missing, due to the lack of modifications in the observed metabolites.

Alternatively, we can provide first promising approaches to uncover the misuse of LEV and HI by obtaining an obviously alternated metabolic profile of the subcutaneously injected insulin compared to the endogenously secreted hormone. DesB30 HI is frequently found in urine samples even from healthy volunteers without exogenous insulin treatment and the concentration strongly correlates to the amount of HI. The comparison of the DesB30 HI / HI ratios in 13 urine samples from healthy persons to the ratios of diabetic patients treated with recombinant HI or LEV is illustrated in Fig. 5.

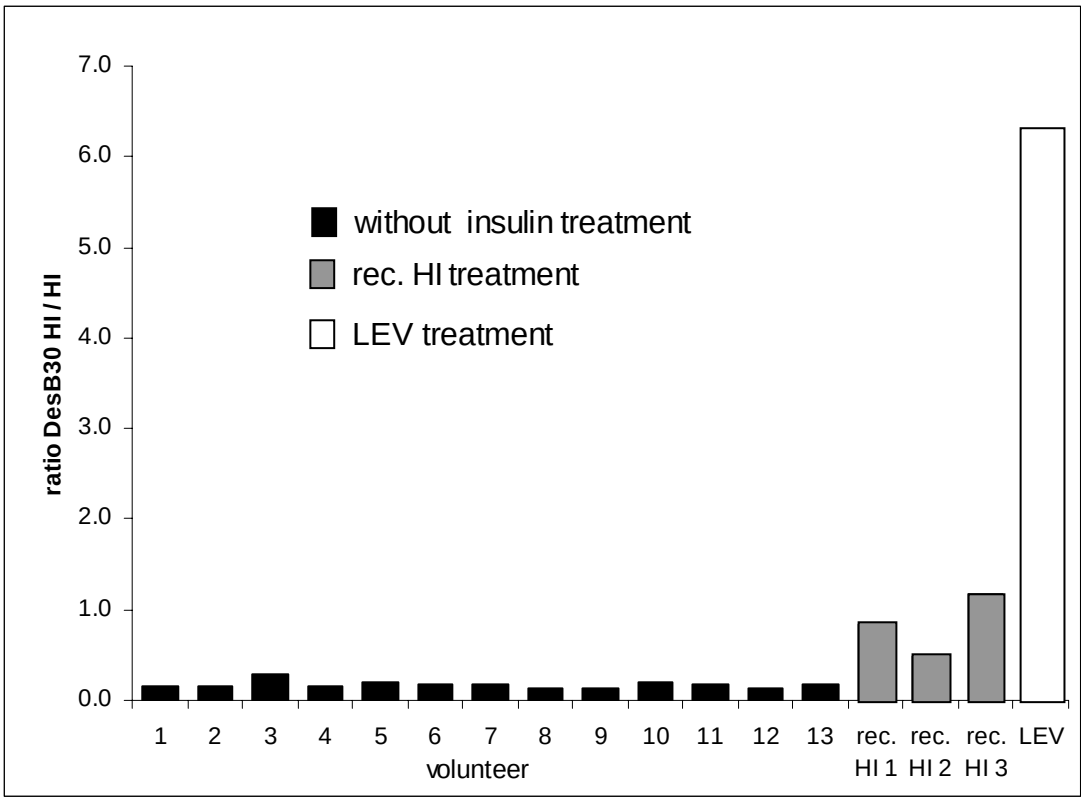

Fig. 5: Metabolic insulin profiles illustrated by DesB30 HI to HI ratios of 13 healthy volunteers (black) without insulin therapy, 3 patients treated with rec. HI (grey) and one patient treated with Levemir (white).

Unless the detailed processes are not finally elucidated, possible explanations for the modified metabolic profile of the long acting analogues were due to prolonged exposure to enzymatic processes in the subcutaneous tissue as well as in circulation. Additionally, for LEV this fact is influenced by the fourfold decreased biological activity and, thus, the absolute amount of injected insulin.

Acknowledgements

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