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Qualitative determination of rapid acting insulin analogues in urine by means of LC-MS/MS

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Introduction

Synthetic insulin analogues were developed to ensure an accommodated injection-to-onset profile for treatment of patients suffering from insulin dependent *diabetes mellitus*[1, 2]. With respect to the pharmacokinetic profile these recombinantly produced peptides are classified in rapid- and long-acting synthetic analogues[3]. The usage of insulin was prohibited by the World-Anti-Doping-Agency for non-diabetic athletes in 1999, but the misuse of these potentially performance enhancing drugs is reported frequently with particularly life-threatening consequences[4 - 7]. At present, several rapid acting insulin formulations, e.g. Humalog (Lispro), Novolog (Aspart) and Apidra (Glulisine), were available purely or as mixtures with recombinant human insulin as long acting component. The primary amino acid sequence of these synthetic insulin analogues slightly differs from human insulin with crucial modifications especially at the C-terminus of the B-chain (Figure 1), whereby the tendency to build hexameric aggregates (subcutaneous) is decreased and lag-phase is shortened consecutively[8].

After mass spectrometric characterisation and qualitative determination in human plasma of the synthetic insulin analogues were described in former studies[9,10], the aim of this project was the development of an analytical procedure to determine these substances in urine, which

is the commonly collected doping control specimen[11]. Renal excretion of intact human insulin as well as its rapid acting synthetic analogues are barely described in literature[12, 13], and the expected concentrations ranged in low femtomole per mL levels, due to efficient adsorption and metabolic degradation in liver and kidney[14, 15], so a mass spectrometric approach with highly sophisticated sample preparation procedure was required. An additional reason to alternate from plasma to urine insulin determination for the rapid acing analogues particularly, was the short half-life of insulins in blood after subcutaneous injection of these substances, so a prolonged detection time window after application was expected[8].

A solid phase extraction (SPE) and immunoaffinity chromatography (IAC) sample preparation, followed by liquid chromatography tandem mass spectrometry was used for a fast and reliable qualitative determination of the target analytes in doping control samples.

Experimental

Materials and Chemicals. OASIS HLB solid phase extraction cartridges (60mg, 3ccm) were obtained from Waters (Milford, MA), acetonitrile (HPLC grade), trisodium phosphate dodecahydrate (p.a.), sodium chloride (p.a.) and acetic acid (glacial) were purchased from Merck (Darmstadt, Germany). Trifluoracetic acid (99+ %), tris(carboxyethyl)phosphine hydrochloride (TCEP-HCl) and bovine insulin were from Sigma (St. Louis, MO). Humalog (Lispro), Novolog (Aspart), Apidra (Glulisine) and recombinant human insulin were supplied by Elli Lilly (Indianapolis, IN), Novo Nordisk (Princeton, NJ) and Aventis (Frankfurt, Germany), respectively. The anti-insulin immunoaffinity gel (0.5 mL/IAC, 10 mg IgG/mL) was obtained from CER (Marloie, Belgium).

Urine samples. All experiments and validation steps were performed with urine samples provided from healthy male and female volunteers. To probe for applicability of the assay, urine samples from diabetic patients and regular doping control samples from elite athletes declaring the treatment with rapid acting insulin analogues were analysed. All samples were stored at 4 °C until analysis.

Stock and working solutions. All solutions were prepared and stored in polyethylene or polypropylene tubes to avoid loss of target analytes on glass surfaces. Stock solutions were stable at 2-8 °C for one week. A solution containing 10 pmol/µL of bovine insulin in 2 % acetic acid was used as internal standard stock solution. Humalog, Novolog and Apidra stock solutions contained 100 pmol/µL in 2 % acetic acid and were diluted freshly before use to a

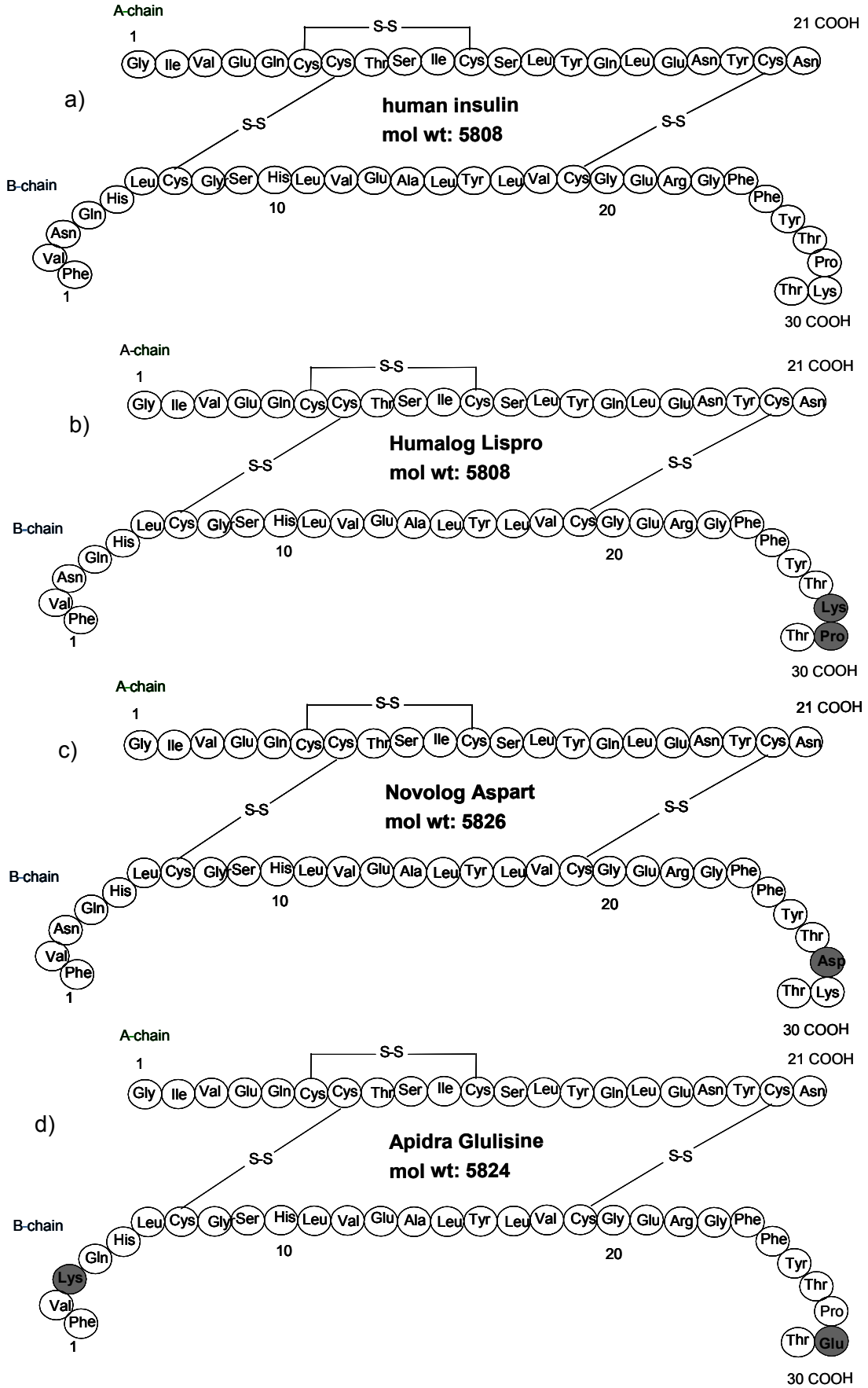

Figure 1: Primary structures of a) human insulin, b) Humalog, c) Novolog and d) Apidra. Modified sites are marked with grey background

final concentration of 0.01 pmol/µL in 2 % acetic acid. These working standard solutions also contained a tenfold carrier-excess of bovine insulin (0.1 pmol/µL), which was added to the Eppendorf tube prior to the target analytes in order to saturate active surfaces.

Phosphate buffered saline (PBS) for IAC is prepared using 120 mM Na_3PO_4 and 0.5 M NaCl in purified water and adjusted to pH 8 with aqueous hydrochloric acid.

Reduction of the disulfide bonds was performed using a 100 mM TCEP-HCl solution by adding 100 µL of this reduction agent to 900 µL of 20 pmol/µL reference solution, followed by incubation for 10 min at 60 °C and dilution with ACN (1:1, v/v).

Mass Spectrometry. Reference mass spectra were measured on an Applied Biosystems Qtrap or Qtrap 4000 mass spectrometer (Foster City, CA) using a syringe pump introducing reference solution (10 pmol/µL) with 5 µL/min. The ion spray voltage was set to 5500 V (positive mode) and collision induced dissociation (CID) was performed with collision offset voltages of 45 V or 75 V for B-chains or intact insulins, respectively, and a collision gas pressure of 4.5×10^{-5} Torr.

Sample Preparation. 25 mL of human urine was fortified with 50 µL of the internal standard stock solution and solid phase extraction (SPE) was performed using OASIS cartridges that were preconditioned with 2 mL of acetonitrile and 2 mL of aqueous acetic acid (2 %). The SPE cartridges were washed with 2 mL of acetic acid (2 %), subsequently. Elution was performed directly to an IAC column, which was filled with 3 mL of PBS, using 1.6 mL of a mixture of acetic acid (2 %) and acetonitrile (1:1; v:v). After addition of another 3 mL of PBS to the IAC column, the mixture was vortexed for 5 s and incubated for 30 min at room temperature with two vortex repetitions after 10 min. The immobilized antigen-antibody-complex was washed 3 times with 3 mL of PBS, and target analytes were eluted onto another OASIS SPE cartridge using 2 x 2 mL of acetic acid (2 %). The SPE cartridge was preconditioned and washed as described above. Elution from the SPE cartridge into a 1.5 mL Eppendorf tube was accomplished using 1.2 mL of a mixture of acetic acid (2 %) and acetonitrile (1:1, v:v). The sample was evaporated to dryness in a vacuum centrifuge at 40°C for approx. 90 min and the residue was reconstituted in 40 µL of a mixture of acetic acid (0.1 %) containing 0.01 % TFA and acetonitrile (72:28, v:v).

To measure the corresponding reduced B-chains, a volume of 2 µL of 100 mM TCEP-HCl solution was added to the reconstituted sample solution, followed by incubation for 10 min at 60 °C prior to injection into the 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 Biosystem Qtrap or Qtrap 4000 mass spectrometer (Foster City, CA). The LC was equipped with a Zorbax 300SB-C18 guard column (1 x 17 mm, 5µm particle size) and a Zorbax 300SB-C18 analytical column (1 x 50 mm, 5µm particle size, 300Å pore size) with an ambient column oven temperature of 40 °C. The mobile phases consisted of 0.1 % acetic acid with 0.01 % 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 and ended at 35 % A after 15 min with a flow rate of 70 µL/min. Subsequently a 23 min equilibration period at starting conditions was added. Injection volumes were 20 µL using the Qtrap and 10 µL for the Qtrap 4000. Both mass spectrometer were operated in positive ion spray mode with a needle voltage of 5500 V. Parameters such as declustering potential, ion trap fill time and entry barrier were optimised for isolation and detection of the fivefold protonated molecules of human insulin, Humalog, Novolog and Apidra. Owing to similar precursor ions for Novolog (*m/z* 1166.2) and Apidra (*m/z* 1165.5), one product ion experiment at *m/z* 1165.5 was sufficient for both analytes with a Q1 resolution of ±0.8 u. Product ion spectra were measured at collision energies of 75 eV utilizing nitrogen as collision gas (4.5×10^{-5} Torr).

Validation items. To proof for method specificity, 10 different urine samples were prepared as described above with no interfering signals at the expected retention times for Novolog and Apidra. For differentiation between Humalog and human insulin the product ions at *m/z* 217 (corresponding to (B)y_2 of Humalog) and the product ion at *m/z* 226 (corresponding to (B)y_3-y_1 of human insulin) were employed as diagnostic ions. The limit of detection (LOD) was determined by a sixfold preparation of an urine sample, that was fortified with 50 pg/mL of each insulin analogue and, considering a signal to noise ratio (S/N) ≥ 3, the relative standard deviations for the sixfold determination of each analyte at the LOD (50 pg/mL) were calculated. To determine the loss of analyte during the preparation procedure, an urine sample were spiked at a concentration of 100 pg/mL and prepared with six replicates as described above. The obtained results were compared to six sample preparations, spiked with 100 pg/mL prior to evaporation in the vacuum centrifuge. Recovery rates were calculated by comparison using the ratios of the product ion scan experiments of the target peak areas to the internal standard of both series. Another six urine samples were fortified with 50, 75, 100, 125, 150 and 200 pg/mL of each reference compound and measured once to demonstrate the linearity of the corresponding signal ratios in this concentration range.

Results and Discussion

Analysis of urine samples. Physiological urinary insulin levels for fasting or non-fasting nonobese subjects have been reported ranging between 20 and 550 fmol/mL[13] and analytical findings of endogenous human insulin in urine within this study complied to these concentrations. Additionally, successful analysis of insulin analogues in urine samples provided from diabetic patients or athletes confirmed the proof of principle, but prognosis of estimated amounts in cheating athletes were difficult. Although, taking into account the athletes intention to accomplish short periods of hyperinsulinamic clamps[5, 16], the assumption is promising, that the working range of the method is sufficient. Figure 3 shows typical chromatograms of product ion scan experiments using the fivefold protonated molecules of a urine sample containing human insulin (endogenous) and Novolog (fortified with 50 pg/mL corresponding to LOD). Distinct signals with a S/N > 3 were obtained at 20.58 min and 20.59 min for human insulin and Novolog, respectively.

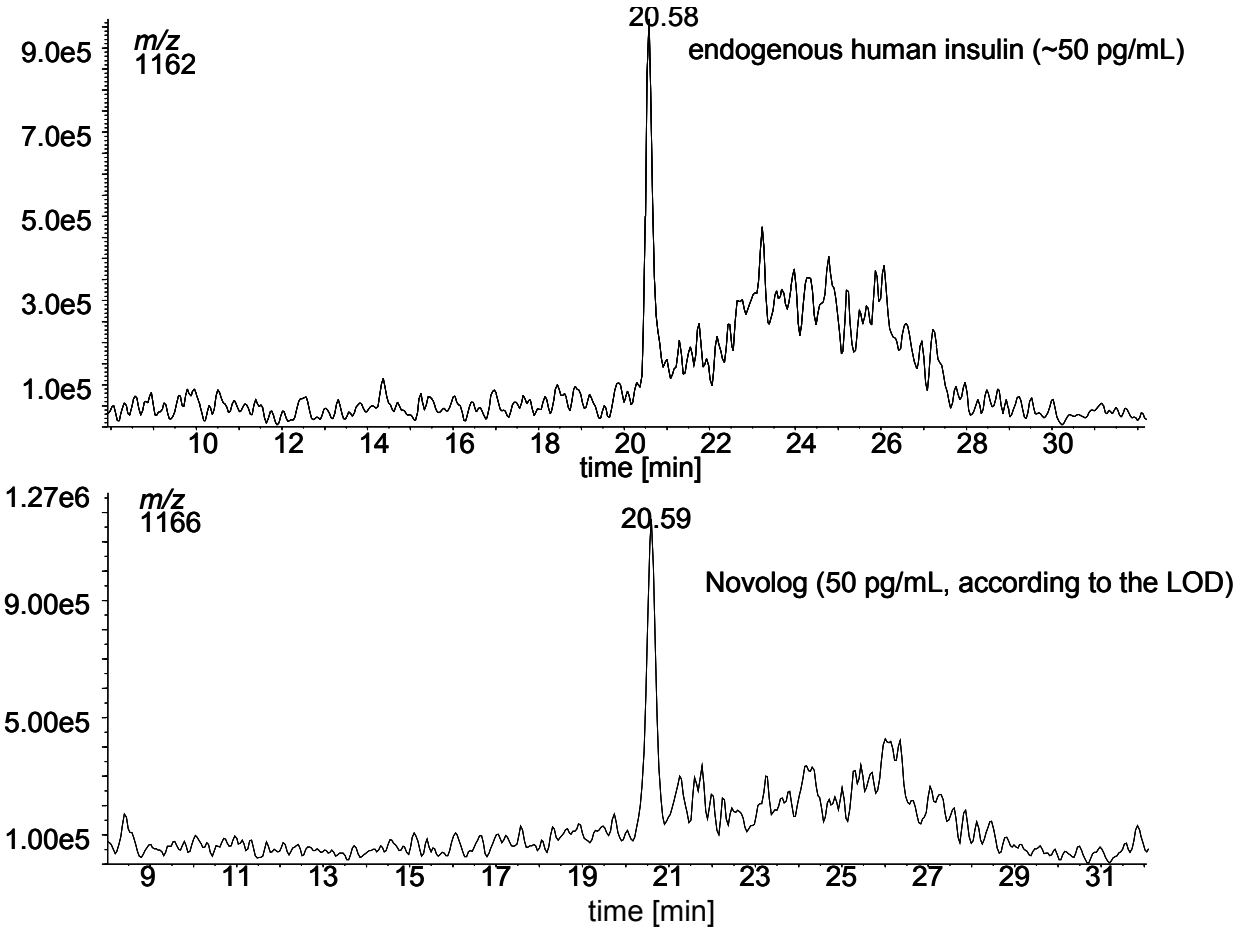

Figure 2: ESI-product ion scan chromatograms of a urine sample containing human insulin (endogenous) and Novolog (fortified with 50 pg/mL) using the fivefold protonated molecule ions $(M+5H)^{5+}$ at *m/z* 1162.5 for human insulin and *m/z* 1166.3 for Novolog.

A product ion scan chromatogram with corresponding product ion scan mass spectra of a regular doping control sample from an athlete suffering from *diabetes mellitus*, who has declared an administration of Apidra is shown in Figure 3. This sample was analysed considering the intact insulin analogue (*m/z* 1165.5) and the reduced B-chain after reduction with TCEP-HCl (*m/z* 862, data not shown), respectively. In the depicted chromatogram an abundant signal at 20.39 min was obtained and in the corresponding mass spectra the characteristic product ions (B)y_3 at *m/z* 346 and (B)y_3-y_1 at *m/z* 227 identified this sample containing Apidra in a concentration of about 25 fmol/mL. Figure 4a shows the product ion scan chromatogram employing the fivefold protonated molecule of intact Humalog and human insulin at *m/z* 1162.5 of an urinary doping control sample provided from an athlete suffering from *diabetes mellitus* and was medicated with Humalog. The presence of the diagnostic ion (B)y_2 at *m/z* 217 in the corresponding mass spectra permitted the differentiation to human insulin despite the same molecular weight and closely related chromatographic behavior. Additionally, unambiguous identification of Humalog was confirmed by analysis of the reduced B-chain only after cleavage of the sequence tag information in the corresponding mass spectra by detecting the doubly charged

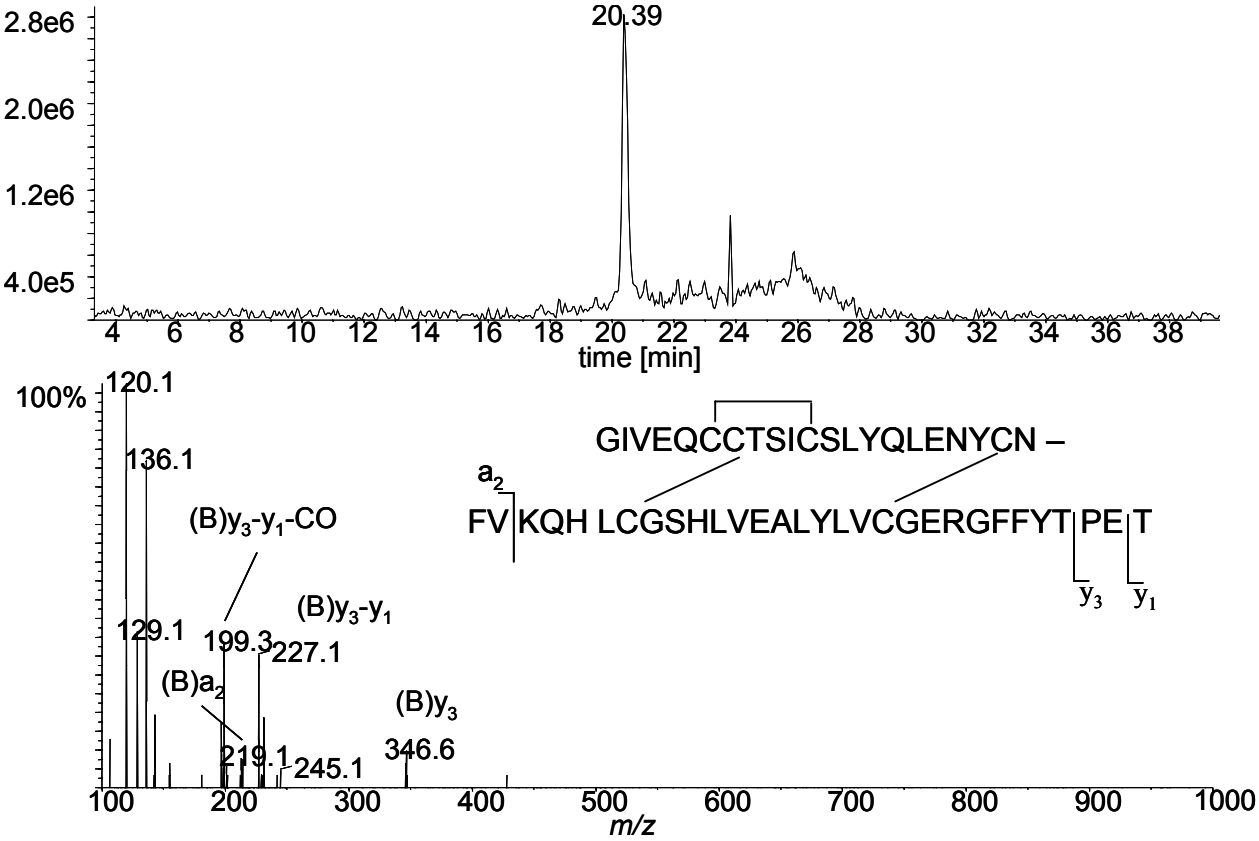

Figure 3: ESI-product ion scan chromatogram (*m/z* 1165.5) with corresponding mass spectra of a regular urinary doping control sample from a diabetic athlete treated with Apidra.

ions b_{10} to b_{17}, beside the proline directed fragment ion[17] at *m/z* 217 as most abundant signal.

Validation results. The procedure was validated for qualitative purposes including the parameters recovery, limit of detection, linearity, precision at the LOD and specificity. The results are summarized in Table 1. Recoveries of about 70-80 % at a concentration level of 100 pg/mL with a precision $\leq$ 20% (n=6) were obtained for all target analytes. Utilizing the chromatographic background noise of ten blank urine samples at the corresponding retention time, a limit of detection of approximately 50 pg/mL was estimated with a signal to noise ratio >3 for each analyte. The endogenous insulin levels dictated the operation range, and the calibration curves were prepared between 50 – 200 pg/mL. The obtained equations were y=1.542x-0.002, r=0.992 for Humalog, y=1.722x+0.004, r=0.980 for Novolog and y=1.523x-0.035, r=0.992 for Apidra. Peak area ratios of the product ions normalized to the internal standard (bovine insulin) were used for the evaluation of linearity and, according to Mandel, linear approximation is permitted. Precisions of less than 20 % (n=6) at 50 pg/mL (LOD) were achieved for all target compounds and specificity was shown by analysis of 10 different urine samples with no interfering signals in the product ion chromatograms for Novolog and Lantus. The absence of the diagnostic ion at *m/z* 217 at the corresponding retention time in the human insulin trace demonstrate the specificity for Humalog, too.

Conclusion

Mass spectrometric analysis of human insulin and its synthetic analogues Humalog, Apidra and Novolog in urine was not accomplished previously due to assumed completely metabolic degradation and concentrations in low femtomole per mL level. The presented method provides a powerful tool to elucidate the misuse of this prohibited substances in commonly collected doping control specimens. Immunoaffinity chromatography, solid phase extraction followed by liquid chromatography/tandem mass spectrometry ensure to obtain fast and reliable results with comprehensive sequence tag information achieved by top down sequencing of the intact insulins and the reduced B-chains, additionally.

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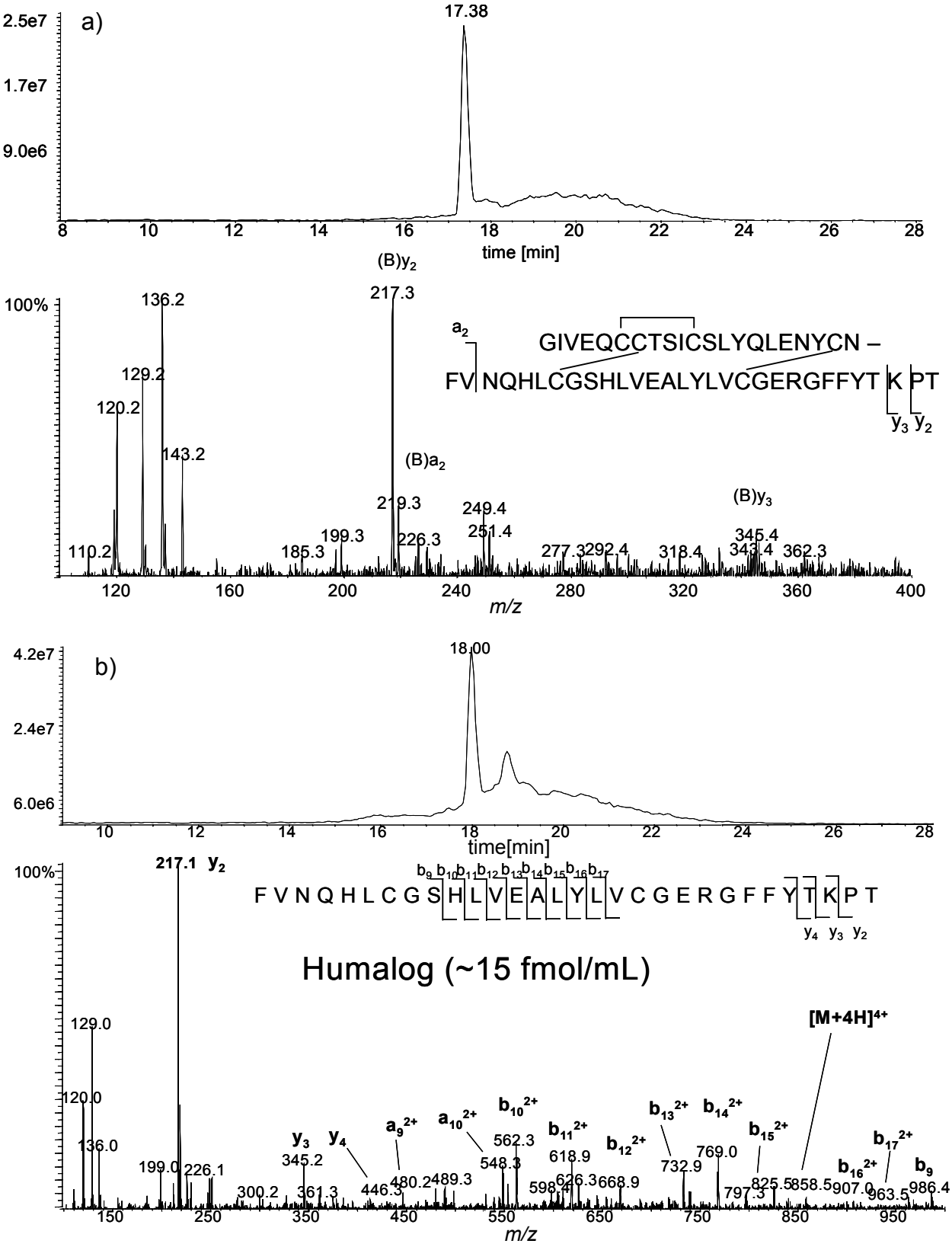

Figure 4: ESI-product ion scan chromatograms with corresponding mass spectra of a) intact Humalog and human insulin (*m/*z 1162.5) and b) the cleaved B-chain (*m/*z 858.5) after reduction with TCEP-HCl, of a regular urinary doping control sample from a diabetic athlete treated with Humalog.

	n	conc. [pg/mL]	result
Specificity	10 blanks	-	no interfering signals
Linearity	6	50 - 200	linear (Mandel); r > 0.990
Limit of detection	-	-	approx. 50 pg/ml (S/N = 3)
Recovery	6 + 6	100	70 - 80 %
Precision at LOD	6	50	< 20 %

Table 1: Validation results, valid for Humalog, Novolog and Apidra

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