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Qualitative determination of synthetic insulin analogues in human plasma by means of LC-MS/MS

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Introduction

The application of recombinant human insulin and its synthetic analogues, such as the fast acting variants Humalog (Lispro) and Novolog (Aspart) or the long acting Lantus (Glargine), has gained enormous importance in the treatment of *diabetes mellitus*. Thus it is an analytical challenge to develop an appropriate method for the reliable determination of these insulins in human plasma, not only for doping control purposes.

Human insulin is an endocrine polypeptide hormone consisting of two peptide chains (A+B) connected by two disulfide bonds at positions $Cys(A)_7$ – $Cys(B)_7$ and $Cys(A)_{20}$ – $Cys(B)_{19}$, with an additional intra-A-chain disulfide bond $Cys\ (A)_6$ – $Cys(A)_{11}$ (see Figure 1 a). The A-chain consists of 21 amino acids, the B-chain has 30 amino acid residues and the molecular weight is calculated with 5808 Dalton (average). The synthetic insulin analogues differ from human insulin only slightly in the amino acid sequence. Humalog, for example, is a rapid acting analogue and differs by the interchange of the proline and lysine residues at position B_{28} and B_{29} (Figure 1 b). This section of the molecule is particularly responsible for the self association to hexameric aggregates and, therefore, for the time of onset, because only monomers posses bioactivity[1]. Consequently the modification of Novolog is a substitution of the proline residue by aspartic acid at position B_{28} (Figure 1 c). In case of Lantus the B-chain is prolonged at the C-terminus by two arginines, and the terminal asparagine residue of the A-chain is substituted by glycine (Figure 1 d)[2]. These modifications cause a shortened or prolonged injection-to-onset profile and lead to short hyperinsulinamic clamps in case of rapid acting analogues or ensure a steady basal supply of long acting Lantus.

Detailed information about the time-concentration-profile in plasma of human insulin, its analogues and mixtures after subcutaneous injection is an important feature for the treatment of the different types of *diabetes mellitus*, for pharmacokinetic or forensic studies as well as for doping control procedures. An abuse of insulin in sport has been reported several times[3] but a concrete evidence of the performance enhancing effect has not been elucidated yet. In particular, the rapid acting analogues are assumed to be misused because of their improved controllability in non-diabetic and non-obese athletes, as compared to recombinant human insulin formulations. Two physiological hypotheses illustrate the possible influences of insulin when it is used as performance enhancing agent[4]. The first one is based on the fact, that insulin increases the concentration of glycogen in muscle cells and provides an increased source of carbohydrates for competition and recovery phases. The second hypothesis describes anticatabolic effects of insulin by inhibition of the protein breakdown in muscles. Generally, an immense risk to incur a hypoglycaemic clamp exits, when insulin is injected in addition to the endogenously formed hormone[3]. All these facts have led to the consequence that insulin and its analogues are on the list of prohibited substances of the International Olympic Committee (IOC) and the World Anti Doping Agency (WADA) since 1999, with an exemption for athletes with a diagnosed *diabetes mellitus*[5]. The assays commonly used for clinical identification are radioimmunoassays (RIA) or enzyme-linked immunosorbent assays (ELISA). They are commercially available and possess variable target selectivities for human insulin, its analogues and some animal insulins[6]. But with regards to the selected epitope, several problems with cross-reactions to precursors (proinsulin) or degradation products of insulin were observed in the past and have led to the development of improved immunoassays with high specificity[7-9] and methods utilizing mass spectrometry[13,14]. In the present study we describe the development of a method for the simultaneous identification of the synthetic insulin analogues Humalog (Lispro), Novolog (Aspart) and Lantus (Glargine) in human plasma beside human insulin by means of immunoaffinity purification, liquid chromatography and tandem mass spectrometry.

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

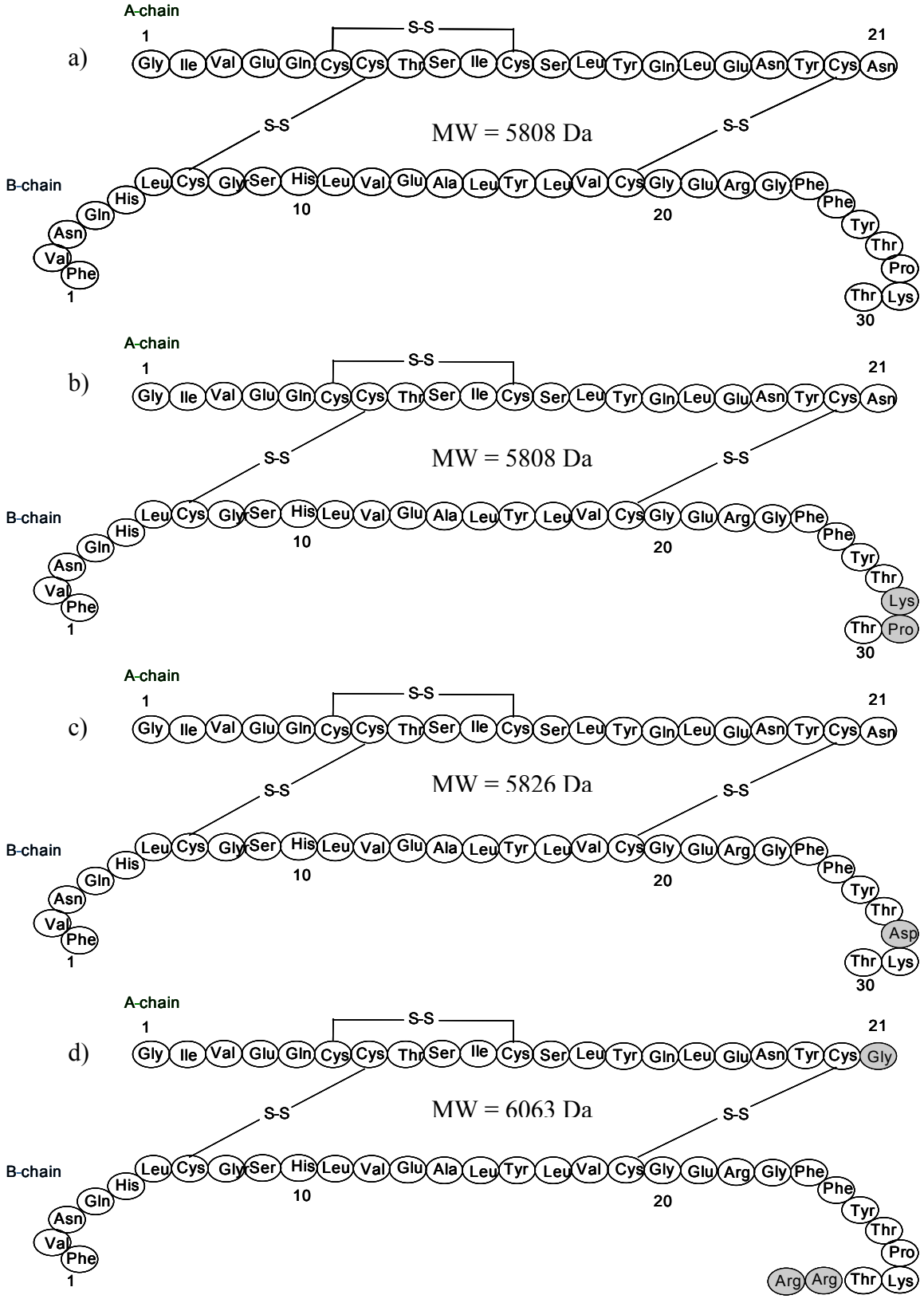

Figure 1: Primary structures of a) human insulin, b) Humalog, c) Novolog and d) Lantus. Modified sites are marked with grey background

hydrochloride (TCEP-HCl), human plasma (lyophilized) and bovine insulin were from Sigma (St. Louis, MO). Humalog (Lispro), Novolog (Aspart), Lantus (Glargine) and recombinant human insulin were supplied by Elli Lilly (Indianapolis, IN), Novo Nordisk (Princeton, NJ), Aventis (Kansas City, MO) and Aventis (Frankfurt, Germany), respectively. The anti-insulin immunoaffinity gel (0.5mL/IAC, 10mg IgG/mL) was obtained from CER (Marloie, Belgium).

Plasma samples. All experiments and validation steps were performed with commercially available plasma, but to show the specificity of the method, 10 different plasma samples of healthy volunteers (obtained from the Department of Cardiology of the German Sports University, Cologne, Germany) were analyzed,.

Stock and working solutions. All solutions were prepared in polyethylene (PE) tubes to avoid loss of target analytes on glass surfaces. Stock solutions were stored at 2-8°C for no longer than 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 Lantus stock solutions contained 100 pmol/µL in 2% acetic acid and were freshly diluted before use to a 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. The 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 HCl.

Reduction of Disulfide Bonds. In order to obtain more detailed information about the fragmentation behaviour, the disulfide bonds of insulin were reduced using 900µL of a 20 pmol/µL insulin solution in 2% acetic acid and 100µL of a 100 mM TCEP-HCl solution by incubation at 60°C for 10 minutes. After dilution with acetonitrile (1:1, v:v) the B-chains were analyzed by ESI-MS/MS.

Mass Spectrometry. To obtain the characteristic product ion mass spectra of human insulin and its analogues the 5-fold protonated molecules were isolated in the first quadrupole (Q1) of the mass spectrometer prior to collision induced dissociation (CID) in the collision cell. Full scan spectra were subsequently measured in the linear ion trap (LIT). Precursor ions were calculated at *m/z* 1147.6 for bovine insulin, 1166.2 for Novolog, 1213.7 for Lantus and 1162.4 for human insulin and Humalog. With respect to the identical molecular weights and thus the identical precursor ions for human insulin and Humalog their identification is performed by diagnostic fragment ions originating from the modified amino acid sequence of the B-chain.

All spectra were measured on an Applied Biosystems Qtrap mass spectrometer (Forster City, CA) using an ESI ion source. Reference compounds were introduced as working solutions by means of a syringe pump at a flow rate of 5 µL/min. The ion spray voltage was 5500 V and CID was performed with a collision energy of 80 eV and a collision gas pressure of 4.5 x 10^{-5} Torr.

Sample Preparation. 2 x 1 mL of human plasma were placed in two 1.5 mL Eppendorf tubes, fortified with 10µL of the internal standard stock solution, vortexed for 5 s and centrifuged for 5 min at 6000 *g*. The supernatants were transferred onto an IAC column filled with 2 mL of PBS. After addition of another 2 mL of PBS the mixture was vortexed for 5 s and incubated for 30 min at room temperature with two vortex repetitions after 10 min. The diluted plasma was eluted from the IAC column while the target analytes were retained by the antigen-antibody-interaction. The immobilized antigen-antibody-complex was washed 3 times with 3 mL of PBS, and insulins were eluted directly onto an OASIS solid phase extraction (SPE) cartridge using 2 x 1 mL of acetic acid (2%). The SPE cartridge was preconditioned with 2 mL of ACN and 2 mL of acetic acid (2%) and washed with 2 mL of a mixture of acetic acid (2%) and ACN (9:1, v/v) after loading with the sample that was eluted from the IAC extract. 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 (6:4, v:v). The sample was evaporated to dryness utilizing a vacuum centrifuge at 40°C for approx. 90 min. The residue was reconstituted in 40 µL of a mixture of acetic acid (0.5 %) containing 0.01 % TFA and acetonitrile (72:28, v:v).

LC-MS/MS. LC was performed on an Agilent 1100 Series high performance liquid chromatograph (Palo Alto, CA) coupled to an Applied Biosystem Qtrap mass spectrometer (Forster City, CA). The LC was equipped with a Zorbax 300SB-C18 guard column (2.1 mm x 12.5 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 0.5% acetic acid with 0.01% TFA (phase A) and a mixture of 0.5% acetic acid with 0.01% TFA and acetonitrile (2:8, v:v) (phase B). The gradient started at 72 % A, ending at 55% A after 15 min with a flow rate of 60 µL/min. Subsequently a 23 min equilibration period was added. The mass spectrometer was 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 5-fold protonated molecules of human insulin, Humalog, Novolog and Lantus. Product ion spectra were measured at collision energies of 80 eV utilizing nitrogen as collision gas (4.5 x 10^{-5} Torr).

Validation items. Specificity was shown by preparing 10 different plasma samples as described above with no interfering signals at the expected retention times for Novolog and Lantus. Differentiation between Humalog and human insulin was obtained with respect to the characteristic product ion at *m/z* 217 of Humalog, which was not detected in the untreated human plasma samples (see also chapter: Results and discussion). The limit of detection (LOD) was determined by comparison of six plasma samples, fortified with 0.5 ng/mL of each insulin analogue and the blank plasma samples measured for specificity. Considering a signal to noise ratio ≥ 3, the relative standard deviations for a six fold determination of each analyte at a concentration level of 0.5 ng/mL were calculated. In order to determine the loss of analyte during the preparation procedure, six plasma samples were spiked at a concentration of 0.8 ng/mL and prepared as described above. The obtained results were compared to six sample preparations, spiked with 0.8 ng/mL prior to the evaporation in the vacuum centrifuge. Recovery rates were calculated by comparison using the ratios of the target peak areas to the internal standard of both series. Another six plasma samples were fortified with 0.5, 0.75, 1.0, 1.25, 1.5 and 2.0 ng/mL of each reference compound and measured once to demonstrate the linearity of the signal ratios in this concentration range.

Results and Discussion

Mass spectrometric characterisation of the intact insulins and the cleaved A- and B-chains. Product ion mass spectra obtained from the five fold protonated molecules $(M+5H)^{5+}$ show small but characteristic fragment ions at collision energies higher than 75 eV[10,11]. Novolog, Lantus and bovine insulin (Internal Standard) differ from human insulin by their molecular weight, so separation via precursor isolation in the first quadrupole is achieved. For Humalog and human insulin, that differ only by the interchanging of two amino acid residues in the B-chain, a sufficient chromatographic resolution on the LC-column was not obtained under the chosen conditions and therefore identification of these compounds required tandem mass spectrometry. The product ion spectra of the intact human insulin and Humalog show common fragment ions at *m/z* 120, 128 and 136, but differentiation of these analytes was possible utilizing the fragment ion at *m/z* 226 (y_3-y_1) for human insulin and the corresponding ion at m/z 217 (y_2) for Humalog. The proposed origin of these diagnostic ions were supported by measurements of the cleaved B-chains, that also gave rise to abundant signals at respective *m/z* values. Figure 2 shows the product ion spectra resulting from the four-fold protonated molecules $(M+4H)^{4+}$ of the B-chains (*m/z* 858.5) of human insulin and Humalog, with the

most abundant fragment ions at *m/z* 226 corresponding to y_3-y_1 of human insulin and *m/z* 217, which was identified as y_2 of Humalog. Both fragments resulted from a typical proline-directed fragmentation, that is described in the literature for various peptides and is related to the secondary amino group of proline in the peptide bone structure[12].

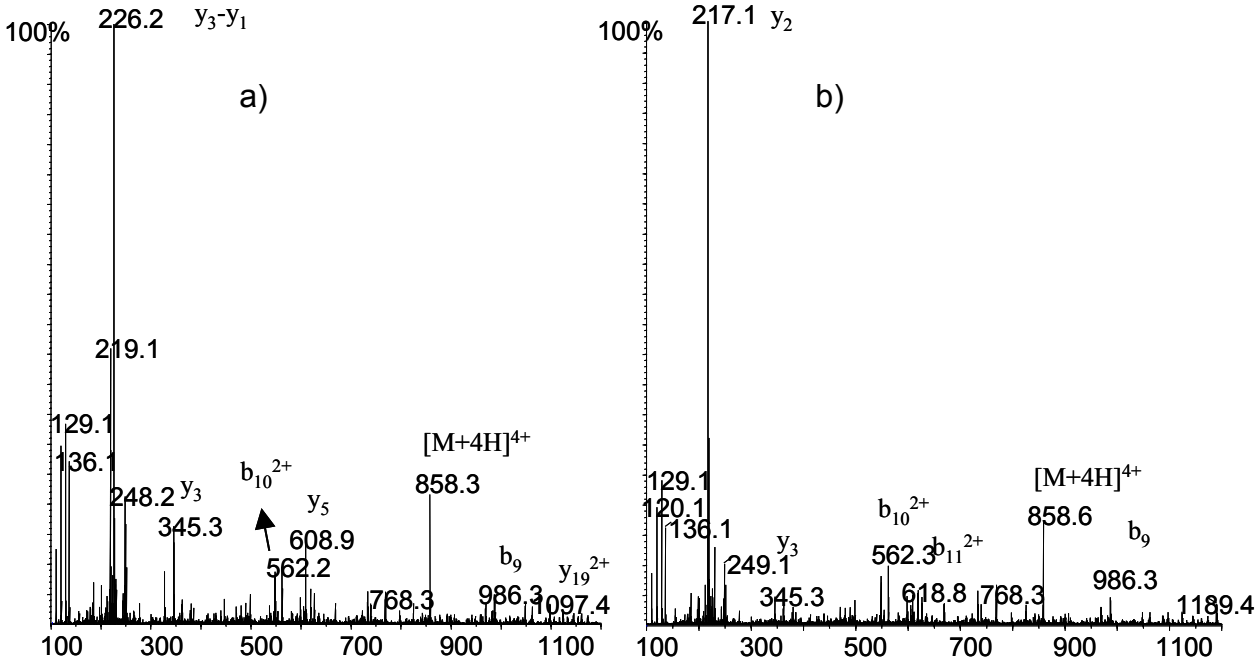

Figure 2: ESI-product ion spectra of the $(M+4H)^{4+}$ at *m/z* 858.5 of the reduced B-chain of human insulin (a) and Humalog (b).

Analysis of Plasma samples. Physiological insulin plasma levels in non obese subjects range from 0.5 - 3.2 ng/mL (normal non fasting subjects) to 0.1 – 0.5 ng/mL (basal fasting status)[13,14]. The non-fasting levels were set as target values for synthetic insulin analogues in doping control samples for non diabetic athletes in the present study. With respect to the lack of information about the amounts and application periods of the synthetic insulins used by cheating athletes, a secure prognosis of the expected levels is not possible. But recent studies describe the anticatabolic effect of the hormone during hyperinsulinamic clamps and therefore determination at endogenous levels were supposed to be sufficient[4]. Figure 3 shows typical chromatograms of product ion experiments using the 5-fold protonated molecules of Novolog and Lantus in plasma samples fortified with 1 ng/mL of the analytes. The corresponding signals were obtained at 22.86 min and 22.44 min.

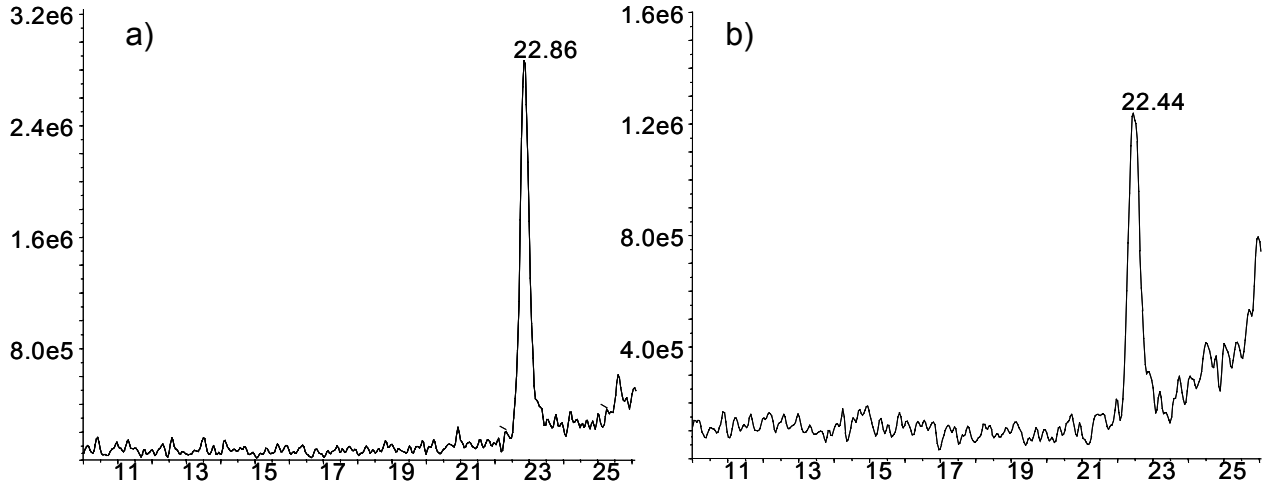

Figure 3: Chromatograms of plasma samples spiked with 1 ng/mL of Novolog (a) or Lantus (b), represented by the product ion scan experiments using $(M+5H)^{5+}$ at *m/z* 1166.2 and *m/z* 1213.7.

The chromatogram of a plasma sample fortified with 0.8 ng/mL of Humalog is shown in Figure 4 a). This sample contains Humalog besides endogenous human insulin, and in the corresponding product ion spectra the diagnostic fragment ion at *m/z* 217 of Humalog is present besides the fragment at *m/z* 226 originating from human insulin. The blank plasma sample in figure 4 b) provided no signal at *m/z* 217, but the fragment at *m/z* 226 that identifies the presence of human insulin only.

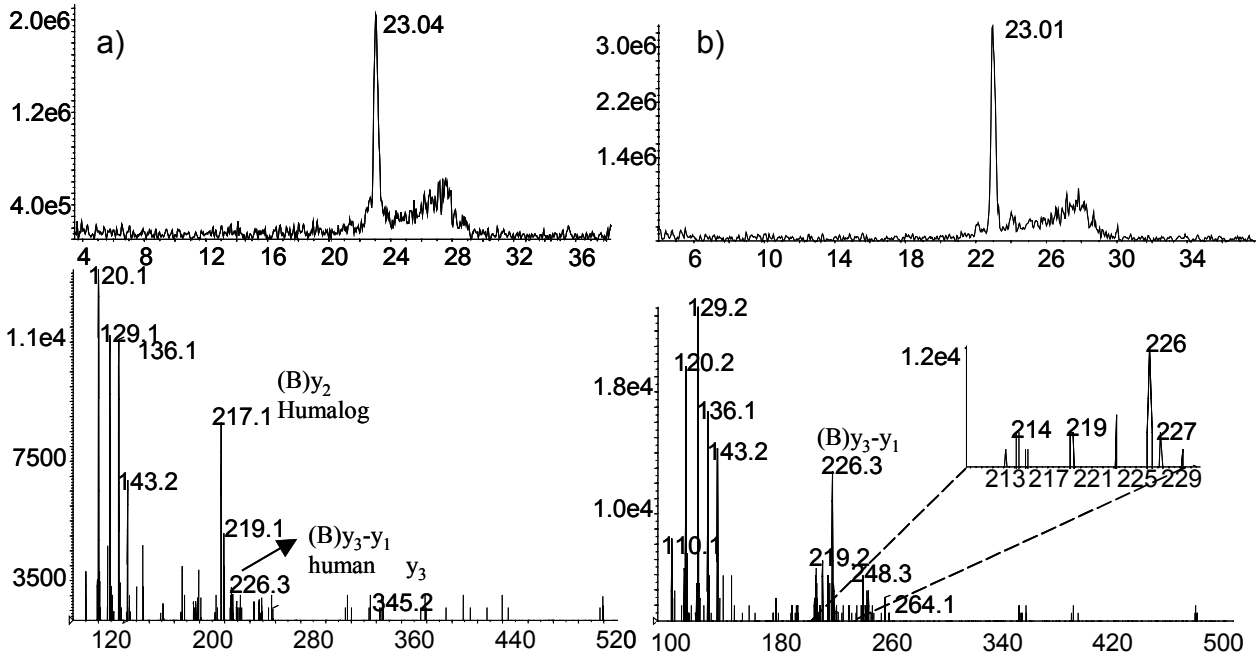

Figure 4: Product ion chromatograms with corresponding spectra of a plasma sample fortified with 0.8 ng/mL of Humalog (a) and a blank plasma sample containing endogenous human insulin only (b).

Validation results. To obtain a common performance evaluation of the procedure, especially for doping control analysis, the method was validated including the parameters specificity, limit of detection, recovery and linearity. The results are summarized in Table 1. Remarkable recoveries > 90% (mean) were obtained at a concentration level of 0.8 ng/mL with a precision $\leq$ 20% (n=6), enabling the usage of 2 mL of plasma only. Utilizing the noise of ten blank plasma samples at the corresponding retention time, the limit of detection of approximately 0.5 ng/mL was calculated with a signal to noise ratio >3 for each analyte. The endogenous insulin levels set the operation range, and the calibration curves were prepared between 0.5 – 2 ng/mL. The obtained equations were y=0.1144x+0.0357, r^2=0.993 for Humalog, y=0.1058x+0.0104, r^2=0.992 for Novolog and y=0.0566x+0.0049, r^2=0.990 for Lantus. Peak area ratios of the product ions normalized to the internal standard (bovine insulin) were used for the evaluation of the linearity and, according to Mandel, linear approximation is permitted for each analyte. Due to the same precursor ion at m/z 1162.4 and simultaneous elution, pooled plasma samples fortified with Humalog contained a constant amount of human insulin. Thus the calibration curve of Humalog is elevated by about 0.3 ng/mL, corresponding to a basal endogenous insulin level in the spiked plasma. Specificity was shown for 10 different plasma 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.

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

	n	conc. [ng/ml]	result
Specificity	10 blanks	-	no interferring signals
Linearity	6	0.5 - 2.0	linear (Mandel); r > 0.990
Limit of detection (LOD)	-	-	approx. 0.5 ng/ml (S/N = 3)
Recovery	6 + 6	0.8	70 - 120 %
Precision at LOD	6	0.5	< 20 %

Conclusion

Regarding the lack of routine procedures to elucidate the abuse of insulin in doping control samples, the present study provides a fast and reliable procedure to determine the synthetic insulin analogues Humalog, Novolog and Lantus in human plasma. The benefit of the combination of immunoaffinity chromatography and tandem mass spectrometry is the exclusion of cross reactivities to endogenous insulin or to precursors or degradation products

and provides an enhanced specificity after sufficient target analyte isolation. The IAC purifies Humalog, Novolog, Lantus and human insulin simultaneously in one separation step, so sample preparation requires approximately 5 hours per sample and multiple sampling utilizing more than one IAC column is possible. All materials are commercial available, so the transfer to other doping control laboratories is feasible.

Acknowledgment

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