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Sensitive and fast identification of urinary human, synthetic and animal insulin by means of nano-UPLC coupled with high-resolution/high-accuracy mass spectrometry

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The double-chain polypeptide insulin and its synthetic (Insulin Glulisine, Insulin Aspart, Insulin Glargine, or Insulin Lispro) or animal analogues (porcine insulin or bovine insulin) are potential performance-enhancing agents in elite sports or potentially effective toxins in forensic science.

The present study demonstrates an analytical method to purify the insulins simultaneously from urine specimens with an approach based on immunoaffinity isolation, using coated magnetic beads (anti-mouse) and a primary anti-insulin antibody (IgG, monoclonal). The extracts were purified sufficiently for separation by means of nano-flow liquid chromatography coupled with nano-scale high-resolution, high-accuracy ESI-MS/MS. Elucidation of collision-induced dissociations with product ion experiments using the fivefold protonated precursor ion of each target analyte enabled all synthetic and animal insulins to be differentiated from their human counterpart, which was particularly important for Lispro, possessing the same molecular mass as human insulin.

The method was fully validated for specificity, limit of detection (LOD, 0.5 fmol/mL), precision (<20%), recovery (approximately 30%) and linearity (2–40 fmol/mL) for all target analytes.

Keywords: Doping; forensic; ion-trap mass spectrometry; orbitrap

Introduction

Recent publications have demonstrated the increasing use of protein-based agents as performance-enhancing drugs and as effective toxins, thus demanding the development of sophisticated analytical tools and approaches in doping control and forensic drug testing.[1–12] One very relevant group of this kind of compounds is the synthetic rapid- or long-acting analogues (for example, Insulin Lispro, Insulin Glulisine, Insulin Aspart, Insulin Glargine), human or animal insulins and their degradation products.[7,13–20]

Electrospray ionization (ESI) and collision-induced dissociation (CID) of insulin and its synthetic analogues is a field that has been well examined in mass spectrometry.[8,21] Nevertheless their differentiation is still a challenge for chromatographic and mass-spectrometric conditions. Glulisine, Aspart, Glargine and porcine insulin differ from human insulin in their amino acid composition and, thus, in their molecular weight, so their identification is possible even in the presence of human insulin (Table 1). In contrast, the elemental composition of Lispro is identical to that of human insulin and differs only in the position of two amino acids. These isomers were distinguished by rather diagnostic but low-mass fragment ions deriving from the C-terminus of the B-chain by CID of the fivefold protonated precursor in a triple quadrupole (QqQ)/linear ion trap mass spectrometer.[4,22] Unfortunately, the direct transfer of this principle from instruments with 'in-space' CID (such as QqQ) to instruments with an 'in-time' MS/MS-option (such as ion traps) is hindered due to the one-third-mass discrimination of small fragments (low-mass cut-off) due to the architecture of these analysers.[23] The present study identifies complementary and alternative options for the differentiation of human insulin and Lispro with diagnostic MS/MS-fragments independent from the MS architecture. This increases the accessibility of the analytical method to a wide range of commonly used mass spectrometers and assists its global distribution as routine procedure or for clinical research and diagnosis.

Classical analytical procedures for doping controls commonly include a set of different analytes that are simultaneously determined in one single screening method to enable the analysis of a large sample number with low workload per sample. Recently developed methods to assay insulin by means of immunoaffinity

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Table 1. Mass spectrometric parameters

Analyte	Molecular weight* [*m/z*]	Dominant charge state	Precursor ion [*m/z*]	Collision offset [%]	Activation Q	Activation time [ms]	Isolation width [*m/z*]	Resolution [FWHM]
Glulisine	5818.64	$[M + 5H]^{5+}$	1165.9	15				
Aspart	5821.61	$[M + 5H]^{5+}$	1165.9	15				
Porcine insulin	5773.63	$[M + 5H]^{5+}$	1156.3	15				
Glargine Met.	5645.75	$[M + 5H]^{5+}$	1130.7	15	0.450	30	1.0	30 000
Lispro	5803.64	$[M + 5H]^{5+}$	1162.5	18				
Human insulin	5803.64	$[M + 5H]^{5+}$	1162.5	18				
Bovine insulin	5726.60	$[M + 5H]^{5+}$	1147.5	15				

* monoisotopic

Human Insulin: GIVEQCCTSICSLYQLENYCN - FVNQHLCGSHLVEALYLVCGERGFFYTPKT

Lispro: GIVEQCCTSICSLYQLENYCN - FVNQHLCGSHLVEALYLVCGERGFFYT**KP**T

Glargine Met.: GIVEQCCTSICSLYQLENYC**G** - FVNQHLCGSHLVEALYLVCGERGFFYTPK

Bovine Insulin: GIVEQCC**A**S**V**CSLYQLENYCN - FVNQHLCGSHLVEALYLVCGERGFFYTPK**A**

Porcine Insulin: GIVEQCCTSICSLYQLENYCN - FVNQHLCGSHLVEALYLVCGERGFFYTPK**A**

Glulisine: GIVEQCCTSICSLYQLENYCN - FV**K**QHLCGSHLVEALYLVCGERGFFYTP**E**T

Aspart: GIVEQCCTSICSLYQLENYCN - FVNQHLCGSHLVEALYLVCGERGFFYT**D**KT

Figure 1. Amino acid sequences from human insulin, Lispro, Glargine Met., bovine insulin, porcine insulin Glulisine and Aspart. Amino acids in bolded black illustrate differences to human insulin.

chromatography (IAC) and mass spectrometry proved to be laborious and were only applicable to a small number of samples due to the use of conventional IAC columns. The purification is highly effective (recovery >80%) and enables the isolation of all target analytes simultaneously;[8] however, the number of samples prepared and analysed per day is limited to about six to eight due to time-consuming sample preparation including one IAC purification and two solid-phase extraction steps. Another recently developed method to determine gonadorelin (GnRH, LHRH) in urine employs a modified purification approach with secondary antibody-coated magnetic particles.[10] This technique enables the simultaneous preparation of up to 30 samples per day and provides a clean extract allowing injection onto nano-flow ultrahigh-pressure liquid chromatography (nano-UPLC) columns.[22] The use of the nanospray ionization (NSI) provides significantly increased sensitivity and thus enables a reduced consumption of urine with concomitantly improved limits of detection (LOD).

The present study describes an effective method to purify all available synthetic insulin analogues, selected metabolites, as well as porcine and bovine insulin from urinary specimens with subsequent analysis by nano-UPLC coupled to an Orbitrap mass spectrometry. The primary structures of all target compounds are illustrated in Figure 1.

Materials and Methods

Coated Dynal beads (anti-mouse IgG) were from Invitrogen (Karlsruhe, Germany). Ultrapure water, acetonitrile, trifluoroacetic acid and formic acid were purchased from Biosolve (Valkenswaard, Netherland). Acetic acid (glacial), acetonitrile (analytical grade), sodium dihydrogenphosphate dihydrate (p.a.), disodium hydrogenphosphate dodecahydrate (p.a.) and sodium chloride (p.a.) were obtained from Merck (Darmstadt, Germany). Monoclonal anti-insulin antibodies (IgG, anti-mouse) were purchased from CER-groupe (Marloie, Belgium). Insulin analogues, Lispro, Aspart, Glulisine and Glargine, were supplied by Eli Lilly (Indianapolis, IN), Novo Nordisk (Princeton, NJ) and Aventis (Kansas City, MO). Porcine insulin, bovine insulin, tris(carboxyethyl)phosphine hydrochloride (TCEP-HCl) and endoproteinase Lys-C from *Lysobacter enzymogenes* were from Sigma (Deisendorf, Germany). Solid phase extraction cartridges OASIS HLB (60 mg, 3 mL) were bought from Waters (Eschborn, Germany). All aqueous buffers and solutions were prepared in MilliQ water.

Urine samples

All urine specimens analysed within the validation process were spontaneous urine samples obtained from healthy male and female volunteers and were stored at 4 °C until analysis. Additionally, one urine sample from an elite athlete who declared the application of synthetic insulin analogue Lispro was analysed.

Standard solutions

All stock solutions were prepared by adequate dilution or weighing of a reference compound to obtain a final concentration of 100 pmol/µL in acetic acid (2%) in polypropylene-tubes. Working solutions were diluted in PP-tubes to a final concentration of 0.1 pmol/µL. Stock solutions were stable for at least one month at 4 °C and working solutions were prepared freshly for immediate use. Bovine insulin was used as an internal standard (ISTD). The

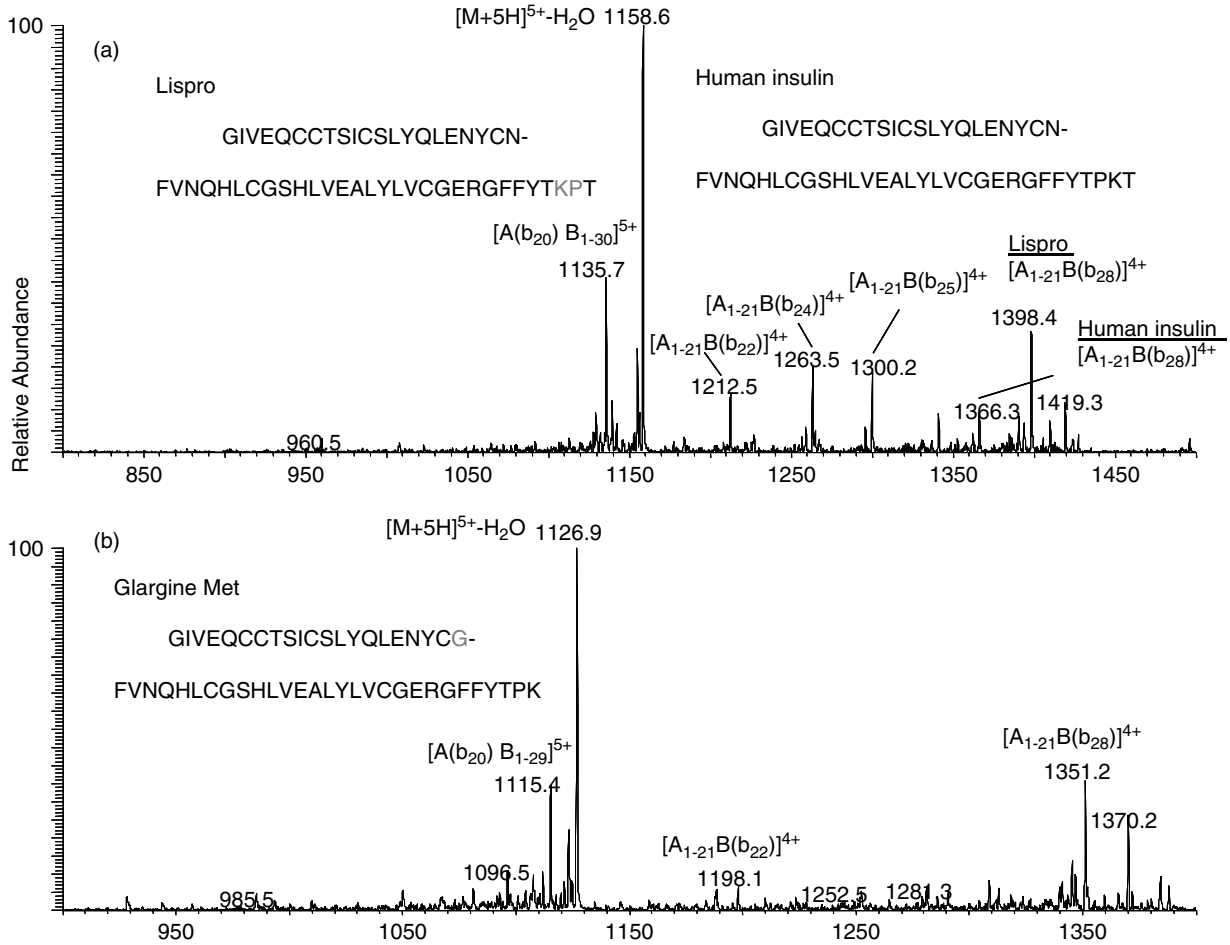

Figure 2. Product ion spectra of the five-fold protonated precursor of (a) mixture of human insulin and Lispro and (b) the metabolite of Glargine. Differences in amino acid sequences compared to human insulin are marked in grey.

Glargine metabolite was produced by enzymatic hydrolysis with *Lysobacter enzymogenes* (Lys-C).[9]

Sample preparation

Acetonitrile (500 µL) and 0.5 pmol (5 µL of ISTD-working solution) of ISTD were added to 5 mL of urine. After gentle mixing the solution was poured onto an OASIS HLB SPE cartridge previously preconditioned with 2 mL of acteonitrile and 2 mL of water. After the sample loading the cartridge was washed with 2 mL of water. Elution with 1.4 mL of a mixture of acetonitrile/water (80/20, v : v) was performed directly into a polypropylene tube. The solvent was evaporated in a vacuum centrifuge with gentle heating (approximately 40 °C). The residue was reconstituted in 500 µL of Phosphate buffered saline (PBS) buffer, followed by the addition of 2.5 µL of anti-insulin antibodies and 35 µL of Dynal magnetic beads suspension. After incubation with gentle stirring for 2 hours at 4 °C, the supernatant was discarded by means of a magnetic separator device and the residual beads were washed twice by consecutive adding, mixing and discarding of 300 µL of PBS. Finally the antigen-antibody aggregate of the target analytes were dissolved by addition of 50 µL of acetic acid (2%) and incubation of 1 min at room temperature. After removing the beads with the magnetic separator, the supernatant was transferred to another PP tube prior to injection into the LC-MS/MS system.

For analysis of B-chains, reduction of the disulfide bonds was accomplished by incubating the sample or reference solution with 10 mM of aqueous TCEP-HCl solution for 10 min at 60 °C.

Liquid chromatography – (tandem) mass spectrometry

The sample cleanup procedure described above allowed the analysis of insulins by means of a nanoUPLC (WATERS Acquity, Milford USA) equipped with a WATERS BEH-130C_{18} (75 µm × 100 mm, 1.7 µm particle size) analytical column and a WATERS Symmetry C_{18} (180 µm × 20 mm, 5 µm particle size) trapping column. Solvent A was purified water and solvent B was acetonitrile, both containing 0.1% formic acid.

After injection of 1 µL of the prepared sample solution, the target analytes were pre-concentrated on the trapping column with 97% of solvent A and a flow rate of 5 µL/min. After 3 min, the flow was diverted to the analytical column at 750 nL/min and the gradient started with an isocratic step for 1 min with 97% of solvent A. The rate of organic solvent B increased to 90% in 21 min, followed by a re-equilibration phase at starting conditions for 13 min. The overall run time was 35 min. Mass spectrometry was performed on a Thermo LTQ-Orbitrap (Bremen, Germany) interfaced with a nanospray source equipped with a coated (standard conductive) fused-silica emitter (New Objective, MA, USA) using positive ionization. Accurate mass measurement was ensured through calibration with the manufacturer's calibration mixture (consisting of

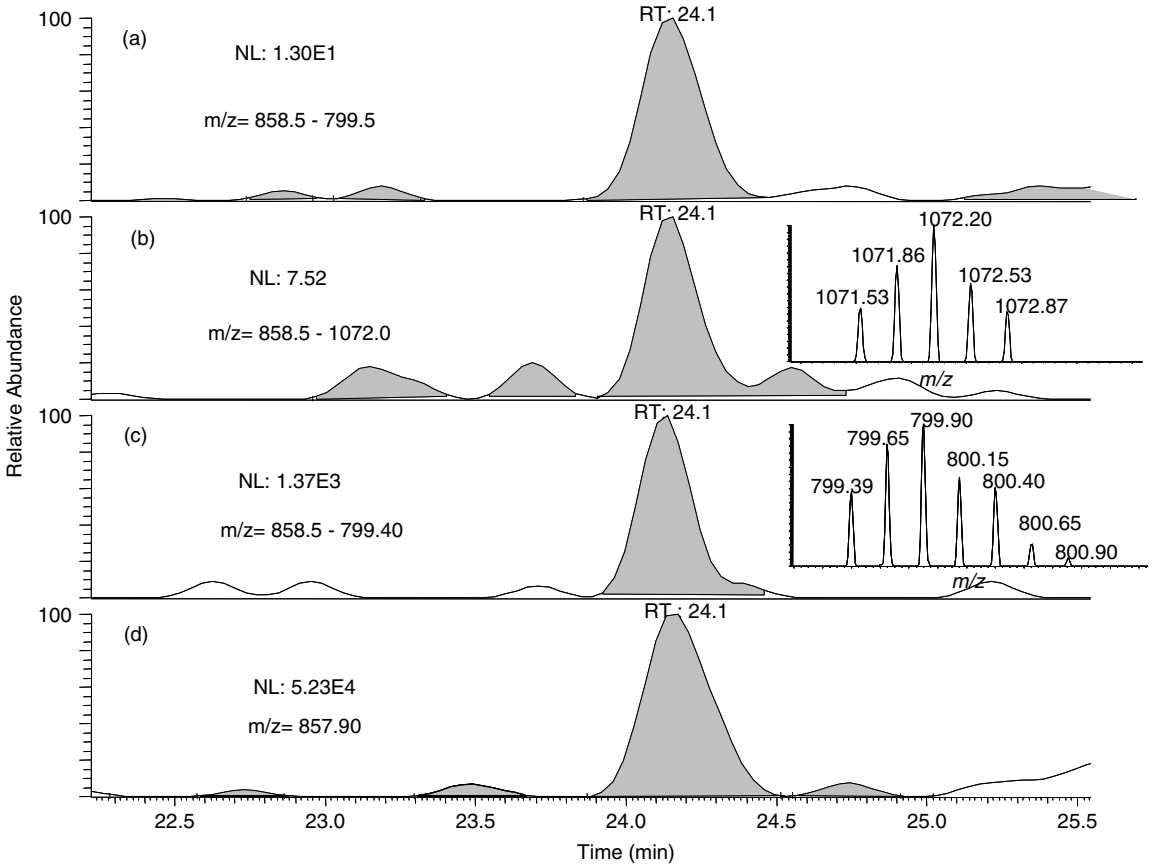

Figure 3. Extracted product ion chromatograms of the reduced B-chain from a fortified urine sample (20 fmol/mL of Lispro) with the diagnostic ion traces obtained from the four-fold protonated precursor at *m/z* 858.5. Diagnostic ions originate from the truncated B-chain as b_{28}^{3+} at *m/z* 1071.53 and b_{28}^{4+}-H_2O at 799.39 (monoisotopic). Traces a) and b) illustrate the extracted product ion traces from the linear ion trap analyser, traces (c) and (d) were acquired in the FTMS. Insets give evidence on the respective charge states.

caffeine, the tetrapeptide MRFA, and Ultramark) and the gas supply consisted of nitrogen (N_2-generator, CMC, Eschborn, Germany) and helium (purity 5.0). The ionization voltage was set to 1.5 kV and the temperature of the transfer capillary was adjusted to 150 °C. Full scan spectra ranging from *m/z* 800 to 1600 were recorded at a resolving power of 30 000 (full width half maximum, FWHM) in the FTMS analyser. Additionally, product ion spectra of the fivefold protonated precursor ions for each insulin were acquired in the linear ion trap. Precursor ions and detailed mass spectrometric parameters are summarized for each analyte in Table 1.

Validation

Validation was performed for qualitative identification of each analyte, considering the parameters specificity, limit of detection (LOD), linearity, precision and recovery, for Lispro, Glulisine, Aspart and porcine insulin. Intact Glargine was not excreted in urine, as reported earlier, and analyses thus focused on the metabolite (DesB30-32) of Glargine.[9] Stability testing of the analytes was not part of the present study but stability at storage conditions of 4 °C and −20 °C was shown earlier.[9]

Specificity

Ten different blank samples (n = 10) from healthy male and female volunteers were prepared and analysed with the addition of ISTD only.

Limit of detection

The lowest level of insulin that is detectable in urine was estimated via signal-to-noise (S/N >3) and verified by six parallel determinations of fortified urine samples (n = 6) at the estimated LOD concentration (0.5 fmol/mL of each insulin).

Linearity

The linearity of the method was determined with fortified samples (n = 6) in concentrations of 2, 4, 10, 20, 30 and 40 fmol/mL. These concentrations correspond to naturally found endogenous human insulin levels in healthy individuals and thus will also fix the general levels for the targeted working range.

Precision (within-day repeatability)

The repeatability of the procedure was proven by the determination of a urine pool (n = 6) fortified with 16 fmol/mL of each insulin and subsequent calculation via peak area ratios of the respective relative standard deviations.

Recovery

The recovery was measured by comparison of the peak area ratios of six urine samples fortified with 32 fmol/mL of each insulin prior to analysis and six urine samples fortified after preparation into the final solutions just prior to injection (n = 6 + 6).

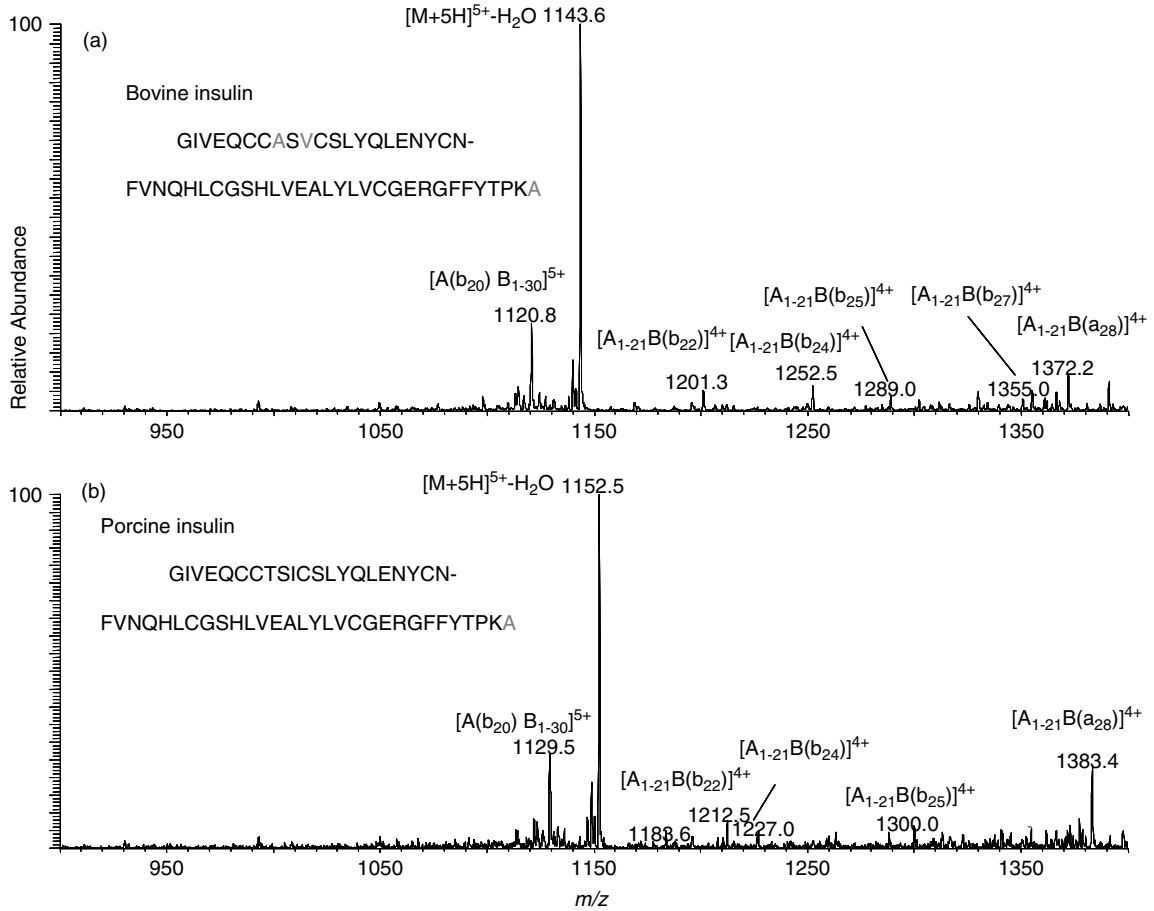

Figure 4. Product ion spectra of the fivefold protonated precursor of (a) bovine insulin and (b) porcine insulin. Differences in amino acid sequences compared to human insulin are marked in grey.

Results and Discussion

In healthy humans, urinary insulin levels range from 10 to 500 fmol/mL, depending on the state of fasting.[24,25] The sensitivity of the method described enables the determination of insulins far below these concentrations and, thus, prolongs the possible detection period after surreptitious application and supports the assaying of minor supplementary amounts of synthetic insulins in specimens of cheating athletes. Furthermore, the improved sensitivity enables the detection of various metabolites of insulin or its synthetic analogues – such as DesB30 human insulin (Figure 1), which was detected in all urine samples – and provides a potentially helpful tool for pharmacological or clinical studies.[9]

Mass spectrometry

Unequivocal differentiation between human insulin and Lispro (Figure 1) was achieved by the fourfold protonated $B(b_{28}{}^{4+})$-product ion at $m/z = 1398.4$ (monoisotopic: $m/z = 1397.89$), which complements the formerly identified $(B)y_2$ at $m/z = 217.1$ originating from the C-terminus of the B-chain.[4,5] This fragment is formed from the fivefold protonated precursor at $m/z = 1162.5$ by the preferred collision-induced cleavage at the amino acid proline (position B_{29}). In contrast, human insulin yielded an abundant fragment $B(b_{27}{}^{4+})$ at $m/z = 1365.87$ (monoisotopic) due to the identical proline-directed fragmentation at position B_{27}. A typical product ion mass spectrum of a mixture of human insulin/Lispro is illustrated in Figure 2 (a). Additional characteristics for the differentiation were provided after mass spectrometric examination of the fourfold protonated precursor at m/z 858.5 of the reduced and isolated B-chain. Product ion experiments yielded the less abundant but diagnostic fragments $B(b_{28})^{3+}$ at m/z 1071.53 (monoisotopic) and $B(b_{28})^{4+}-H_2O$ at m/z 799.40 (monoisotopic).

In Figure 3, extracted product ion chromatograms of a fortified urine sample (20 fmol/mL of Lispro) after reduction of disulfide bonds and MS/MS analysis of the fourfold protonated precursor at m/z 858.5 are shown. Data was acquired in the linear ion trap as well as in the FTMS analyser yielding abundant signals at 24.1 min. The respective blank sample did not produce interfering signals originating from human insulin at the expected retention time.

All other synthetic or animal insulins were readily distinguished from human insulin by their molecular mass and their identification was accomplished in accordance with established recommended criteria for peptides and proteins in doping control or forensic science.[26] Typical linear ion trap product ion spectra (low resolution) of Glulisine, Aspart, porcine insulin and the metabolite of Glargine are shown in Figures 2, 4 and 5. All MS/MS spectra contained an abundant signal $[M+5H-H_2O]^{5+}$, which served as a sensitive diagnostic product ion for screening purposes. Figure 6 illustrates the extracted product ion chromatograms of a urine sample fortified with 0.5 fmol/mL of each insulin. The product ion

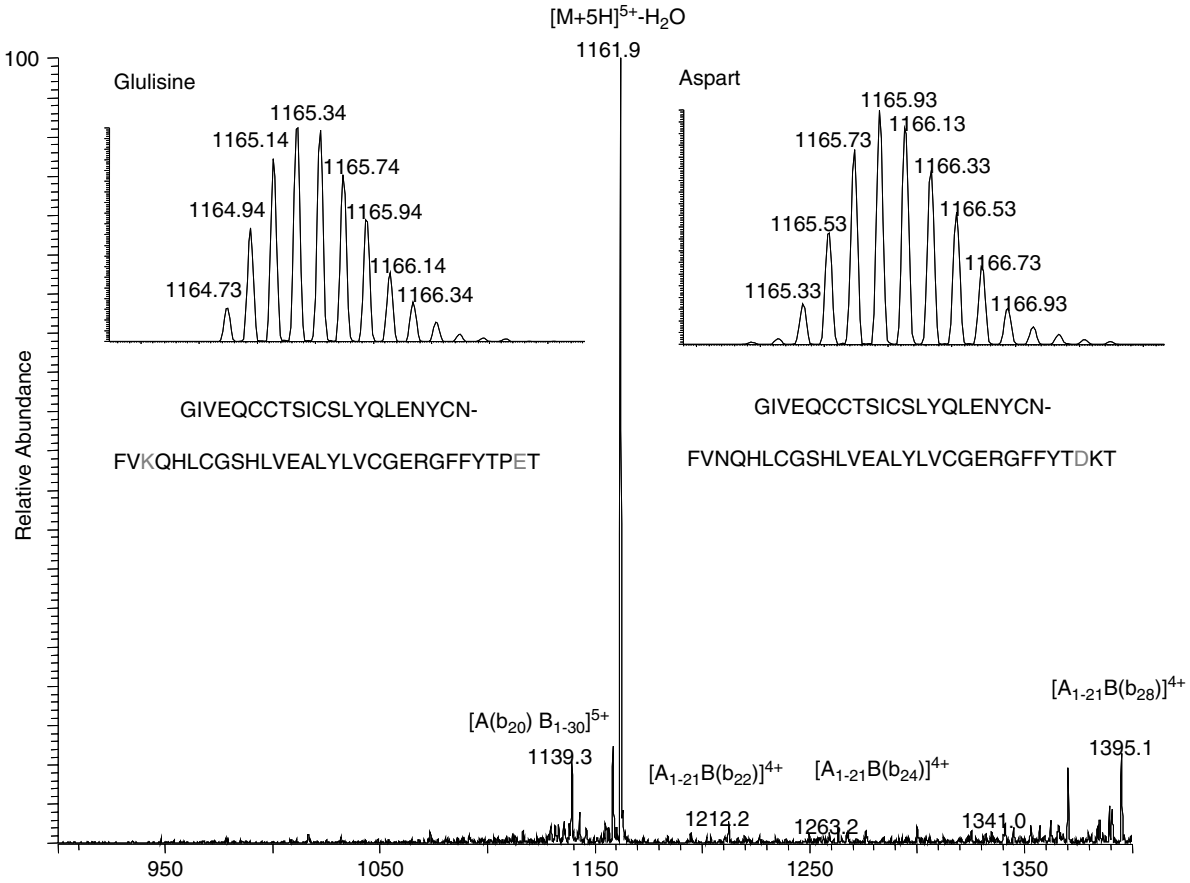

Figure 5. Product ion spectra of the five-fold protonated precursor at *m/z* 1165.9 of a mixture of Glulisine/Aspart. Differences in amino acid sequences compared to human insulin are marked in grey. The insets illustrate the possibility to distinguish the two analytes in the FTMS full scan by accurate mass measurement.

scan experiment at *m/z* 1165.9 in the linear ion trap covered both Glulisine and Aspart due to very similar *m/z*-values of their fivefold protonated precursor ions, averaging *m/z* 1165.5 and 1166.2, respectively. The differentiation of the species was supported by the simultaneously acquired full-scan mass spectra using the high resolution/high accuracy feature of the Orbitrap analyser (Figure 3).

In contrast to these insulin derivatives, the abundant signal at 23.4 min for the ion transition at *m/z* 1162.5/1158.8, namely $[M+5H-H_2O]^{5+}$, was not specific for Lispro due to the presence of coeluting endogenous human insulin (Figure 7). Instead, the additional extracted product ion chromatogram at *m/z* 1162.5/1398.5 was used for the identification of Lispro.

The general performance of the method was evaluated considering the detection of endogenous insulin in each sample even at basal levels in the fasting state. But in order to examine the transfer of the approach to exogenous insulin after application, a urine specimen of an elite athlete who stated the use of Lispro was analysed. Figure 6 shows the product ion chromatograms with the diagnostic ion trace at *m/z* 1662.5/1398.5 and the respective product ion spectra below.

Validation

Main characteristics of the procedure were proven with a validation under consideration of qualitative identification purposes, and the results obtained are summarized in Table 2. Evaluations were calculated considering the peak area ratios to the ISTD of diagnostic ion traces for Glulisine/Aspart, porcine insulin, metabolite of Glargine and Lispro.

Specificity

None of the ten urine samples collected from healthy male and female volunteers in different fasting states provided interfering signals in the product ion chromatograms at the respective retention times. In all samples, the endogenously produced human insulin as well as known metabolites (for example, DesB30 human insulin) were detected but did not interfere with the analysis.

Limit of detection (LOD)

The LOD was estimated for each target analyte at approximately 0.5 fmol/mL by evaluation of the signal-to-noise ratio (S/N). The subsequent determination of six replicates of fortified urine samples at this level provided an adequate S/N >3 for the diagnostic ion trace and relative standard deviations of the peak area ratios of 19–24% (see Table 2).

Linearity

Linearity was tested in concentration steps of 2, 4, 10, 20, 30 and 40 fmol/mL and provided coefficients of correlation of 0.985–0.995 (see Table 2). Linear approximation in this range was permitted for all target insulins according to *Mandel*.[27]

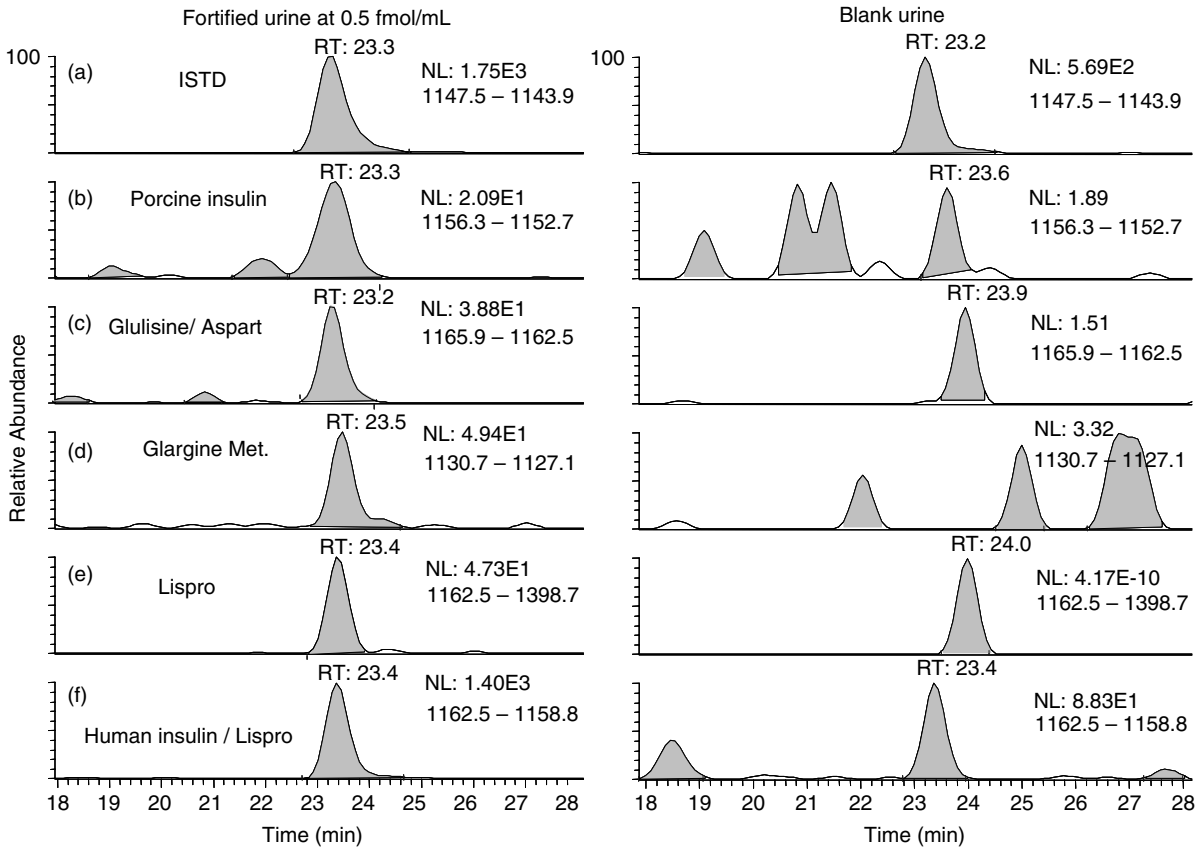

Figure 6. Extracted product ion chromatograms of a fortified urine sample (at LOD 0,5 fmol/mL, left column) and a blank urine (right column) of each analyte with the respective diagnostic ion traces for the (a) ISTD (bovine insulin), (b) porcine insulin, (c) combined trace for Glulisine/Aspart, (d) metabolite of Glargine, (e) Lispro and combined trace for (f) Human insulin/Lispro.

Table 2. Validation results

Analyte	Ion transition [*m/z*]	Precision at LOD (n = 6) [fmol/mL]	Precision at LOD (n = 6) [%]	Recovery ($n = 6 + 6$) [%]	Precision ($n = 6$) [%]	linearity ($n = 6$) Slope	linearity ($n = 6$) Intercept	linearity ($n = 6$) r
Glulisine/Aspart	1165.9/1162.3	0.5	24.1	32.7	17.2	140.34270	1.17347	0.9947
porcine insulin	1156.3/1152.7	0.5	18.9	32.6	10.6	534.27482	−0.83415	0.9982
Glargine Met.	1130.7/1127.1	0.5	22.5	36.3	8.0	165.12246	2.76417	0.9850
Lispro	1162.5/1398.7	0.5	23.1	30.7	18.8	755.21393	1.52942	0.9889
ISTD (bov. Insulin)	1147.5/1143.9	–	–	–	–	–	–	–

Repeatability

The repeatability of the method was calculated by the relative standard deviations from a sixfold determination of a fortified urine pool at 16 fmol/mL. The relative standard deviations of the peak area ratios were calculated with 17% for Glulisine/Aspart, 11% for porcine insulin, 8% for the metabolite of Glargine and 19% for Lispro.

Recovery

The recovery rates were strongly affected by the loss of target analytes during the sample preparation procedure, thus the recovery was evaluated with addition of targets (at 32 fmol/mL) before and after sample preparation. Comparison of peak area ratios yielded recoveries of 33% for Glulisine/Aspart, 33% for porcine insulin, 36% for the metabolite of Glargine and 31% for Lispro. The relative standard deviations were below 15% for all insulins.

Concluding Remarks

The present study was undertaken to address the problem of handling the growing number of samples in this analytical field. The use of antibody-coated magnetic beads enabled chromatography and ionization at the nano-scale level due to very clean extracts and led to a considerable increase in sensitivity by a factor of at least 20 compared with earlier approaches. The newly characterized mass spectrometric fragmentation behaviour of human, animal and synthetic insulins under in-time MS/MS conditions enabled the identification of Glulisine, Aspart, the metabolite of Glargine, porcine insulin and in particular of Lispro

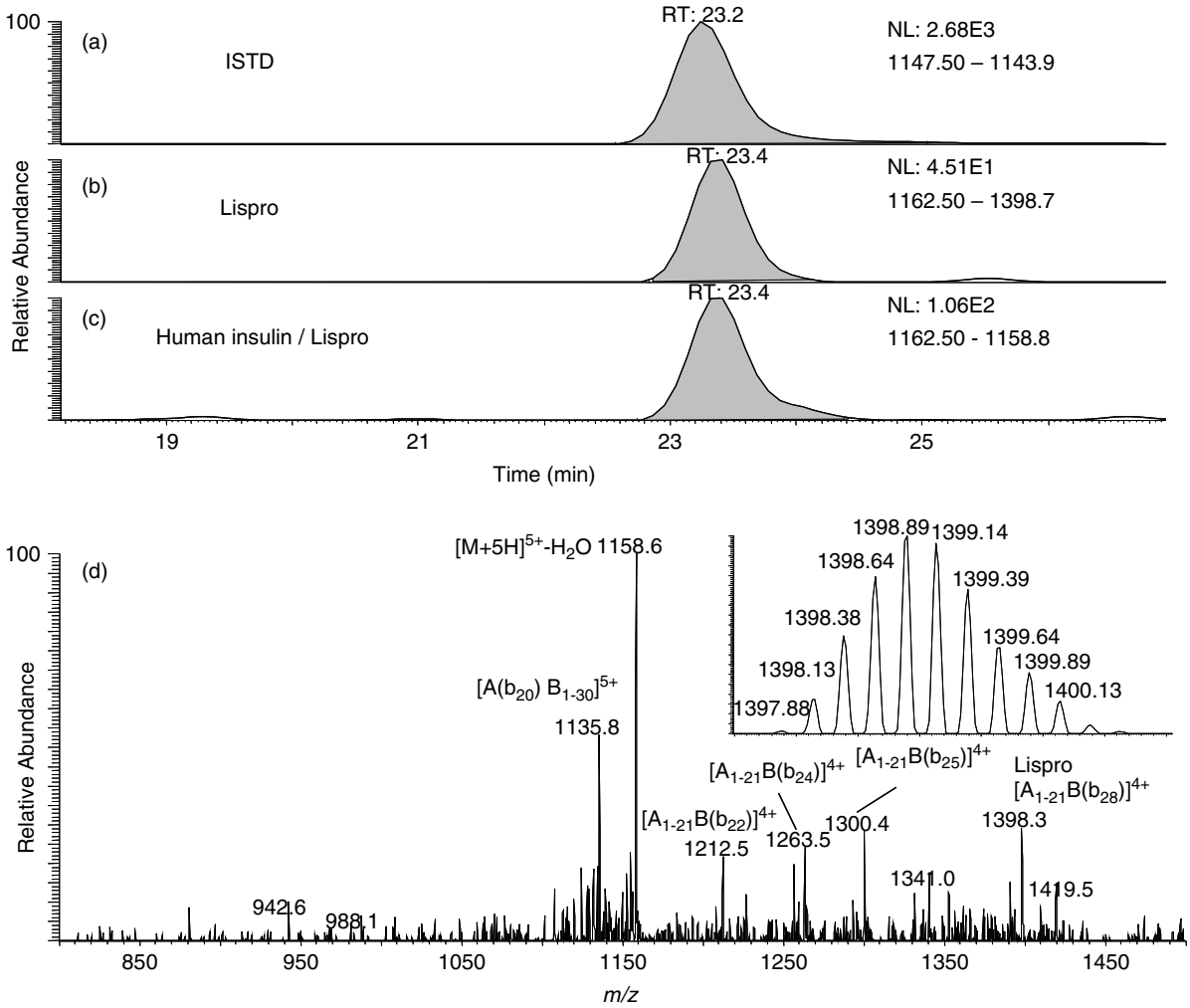

Figure 7. Extracted product ion chromatograms of (a) ISTD, (b) diagnostic ion trace for Lispro, (c) combined human insulin/Lispro ion trace and (d) respective product ion spectra obtained from an elite athlete after application of the rapid acting synthetic insulin Lispro. The inset in the spectra illustrated the charge state of the diagnostic product ion at *m/z* 1397.88 (monoisotopic) acquired in the FTMS analyser.

in presence of human insulin. The simultaneously acquired high resolution/high accuracy full scan data provided highest quality for confirmatory purposes and identifies unambiguously the diagnostic MS/MS fragments for further low resolution screening procedures.

The validated method is appropriate to analyse up to 20 urine samples per day and all chemicals, reagents and instruments used were commercially available; hence routine application and method transfer to other laboratories is not hindered.

Acknowledgements

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