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Mass Spectrometric Identification of Synthetic Insulins

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Introduction

The determination of synthetic rapid- or long-acting insulins, e.g. Humalog® (Lispro) and Novolog® (Aspart) or Lantus® (Glargine), respectively, is necessary in doping control analysis as these peptide hormones are banned according to the list of prohibited substances and methods of doping[1] established by the International Olympic Committee (IOC) and World Anti Doping Agency (WADA). The rapid- or long-acting insulins differ only slightly from human insulin (Fig. 1A), e.g. in case of Humalog® the proline and lysine residues at B_{28} and B_{29} are switched in position (Fig. 1B), and Novolog® comprises an aspartic acid residue at position B_{28} instead of a proline (Fig. 1C).[2] These modifications are done in order to reduce the affinity to self-association of insulin forming hexamers[3], because biological activity of insulin is present only as monomers. Thus, the bioavailability of insulin after subcutaneous injection is accelerated compared to preparations of recombinant human insulin.[4] In contrast, Lantus® is a long-acting insulin analogue with an exchange of the A_{21} asparagine by a glycine, and it contains two additional arginines at the C-terminus of the B-chain (Fig. 1D). Compared to human insulin, an elevated isoelectric point is obtained, which causes a decreased solubility of the drug at physiological pH values and thus a microprecipitation of glargine after subcutaneous injection. The absorption from the injection site is delayed and the duration of action extended[5,6,7]. Different types of immuno assays, such as radioimmuno assay (RIA), enzyme linked immuno sorbent assay (ELISA) or microparticle enzyme immuno assay (MEIA) are commonly employed for identification and quantitation of human and animal insulin[8], since the first assay was introduced by Yalow and Berson in 1959[9]. Nevertheless,

several problems were observed in the past concerning the accuracy of immunoassays, based on cross reactivities to either precursors (such as proinsulin or split proinsulin), degradation products of insulin or analogues to human insulin, e.g. originating from synthetic analogues or animal sources. Although these disadvantages have mainly been overcome with improved immunoassays[10,11,12], several applications utilizing mass spectrometry have been developed for a quantitative analysis of human insulin, its precursors and animal analogues.[13,14,15]

In the present study, we demonstrate the possibility to identify the synthetic analogues of human insulin Humalog (Lispro), Novolog (Aspart) and Lantus (Glargine), in human plasma at levels of 3-10 pmol/mL by means of liquid chromatography interfaced to tandem mass spectrometry.

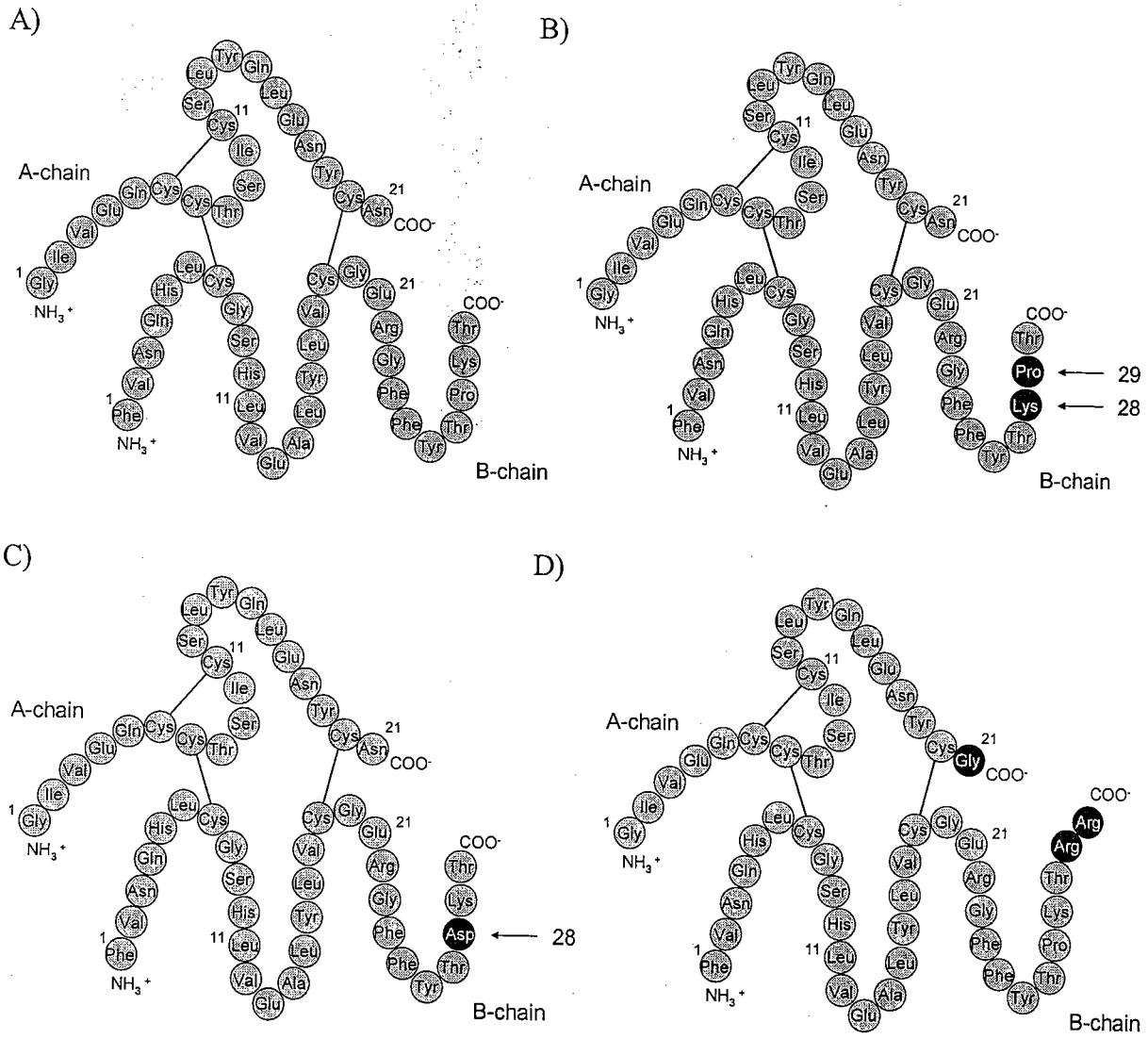

Figure 1: Structure images of human insulin (A), Humalog (B), Novolog (C), and Lantus (D).

Experimental

Chemicals and materials. Chromabond C-18 cartridges (500mg) were obtained from Macherey-Nagel (Düren, Germany). Acetonitrile (HPLC grade) and acetic acid (glacial) were purchased from MERCK (Darmstadt, Germany), and bovine and recombinant human insulin were from SIGMA (St. Louis, MO). Tris(carboxyethyl)phosphine (TCEP-HCl) was obtained from Pierce (Rockford, IL), and the insulin analogues Humalog®, Novolog® and Lantus® were supplied by Eli Lilly (Indianapolis, IN), Novo Nordisk (Princeton, NJ) and Aventis (Kansas City, MO), respectively. Ultrafree®-MC (30K) filtration devices were obtained from Millipore (Bedford, MA). Plasma specimens pooled from eight different healthy volunteers were obtained from the Institute of Cardiology and Sports Medicine of the German Sport University, Cologne, Germany.

Sample preparation. A) intact insulins: Plasma samples of a volume of 1 mL were fortified with the internal standard bovine insulin at 50 pmol/mL, and with the reference compounds Humalog, Novolog and Lantus at concentrations of 3, 10, and 60 ng per mL (corresponding to 0.5, 1.6, and 10 pmol per mL, respectively). The subsequent extraction of the insulin analogues from the biological matrix was adapted from the protocol published by Darby et al.[20] Briefly, the plasma specimen was diluted by an equal amount of 50 mM acetic acid and transferred onto a C-18 column, which was pre-conditioned with 2 mL of acetonitrile followed by 2 mL of 50 mM acetic acid. The loaded sample was washed twice with 2 mL of 20% acetonitrile in 50 mM acetic acid, and the analytes were subsequently eluted with two portions of 2 mL of 40% acetonitrile in 50 mM acetic acid. The combined fractions were dried in a reagent tube *in vacuo* at 60°C, the residue was reconstituted in 100 µl of 0.06 M hydrochloric acid, and passed through an Ultrafree®-MC (30K) filtration device. A volume of 10 µl of the filtrate was injected into the LC-MS system.

B) reduced disulfide bonds: In order to obtain more detailed mass spectrometric information on the analytes, disulfide bonds of insulins were reduced by means of TCEP-HCl. Here, samples were reconstituted in 100 µl of 10 mM aqueous TCEP-HCl after solid-phase extraction and evaporation, incubated at 60°C for 15 min, evaporated to dryness, redissolved in 100 µl of 0.06 M HCl, and filtered through a 30 kDa cut-off membrane. A volume of 10 µl of the filtrate was injected into the LC-MS system.

Liquid Chromatography-Mass spectrometry. Mass spectra were recorded on an APPLIED BIOSYSTEMS (Foster City, CA) QStar Pulsar-*i* QqTOF instrument and an API 2000 triple quadrupole mass spectrometer. The latter was interfaced to an Agilent 1100 Series liquid chromatograph equipped with a Zorbax 300SB C_{18} column (i.d. = 2.1 mm; length = 50 mm; particle size = 3.5 µm, Agilent Technologies, Waldbronn, Germany). The eluents used were A: 0.2% acetic acid containing 0.02% TFA, and B: 80% acetonitrile/20% 0.2% acetic acid containing 0.02% TFA. The flow rate was 300µL/min, and a gradient was utilized from 5% B to 100% B in 9 minutes. The column was finally reequilibrated for 5 min. Nitrogen was used as collision gas at 3.33e-3 Pa (2.5e-5 torr), the ion spray voltage was set to 5500 V and the declustering potential to 25 V. Reference product ion spectra of every analyte were obtained by direct introduction of a solution containing 10 pmol/µl of reference material (dissolved in 50% acetonitrile, 0.1% formic acid) into the mass spectrometer without HPLC by means of nano-electrospray capillaries (Protana, Denmark).

Results and Discussion

Peptides and proteins (such as insulin) commonly generate multiply charged molecules upon electrospray ionization. In Figure 2a, a full scan ESI-spectrum of human insulin is presented, containing the five-, six-, and seven-fold protonated molecules at *m/z* 1162.4, 968.9, and 830.6, respectively. Depending on different parameters of ionization, e.g. declustering potential, source design or flow rate, distinct charge states are predominant as observed with $(M+6H)^{6+}$ in case of human insulin analyzed on a QStar Pulsar-*i* QqTOF instrument equipped with a nano-electrospray ion source.

In plasma samples prepared according to the protocol described above, intact synthetic insulins were determined by their multiply protonated molecules at concentrations of 10 pmol/mL as shown in Figure 2b, and an estimated detection limit of 3 pmol/mL was accomplished by means of LC-MS/MS. Owing to average plasma insulin concentrations of 0.3-2.0 ng/mL corresponding to 0.05-0.3 pmol/mL, these detection limits are not sufficient and need to be improved by more suitable instrumentations such as more sensitive mass spectrometers and/or micro/capillary liquid chromatography.

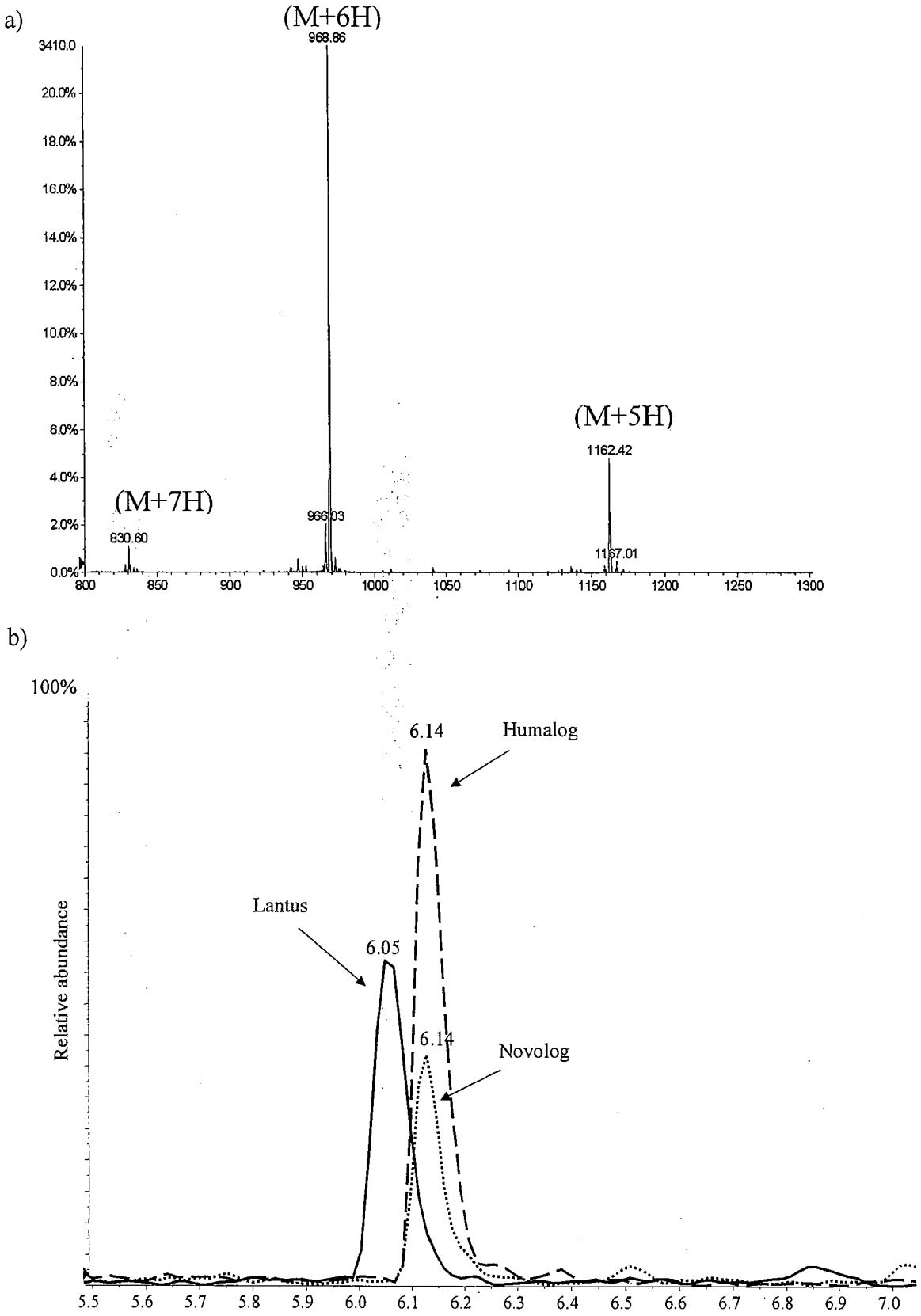

Figure 2: a) ESI full scan spectrum of human insulin generating the multiply charged molecules $(M + 5H)^{5+}$, $(M + 6H)^{6+}$, and $(M + 7H)^{7+}$ at *m/z* 1162.4, 968.9, and 830.6,

respectively; b) extracted ion chromatogram of a plasma sample fortified with Humalog (*m/z* 1162), Novolog (*m/z* 1166) and Lantus (*m/z* 1213) at 10 pmol/mL. Insulins were analyzed as intact proteins.

The collisionally cactivated dissociation (CAD) of multiply charged molecules of insulin gives rise to a product ion spectrum shown in Figure 3, containing structural information based on various singly or multiply charged b- or y-fragment ions. In case of xenobiotics, i.e. non-endogenous compounds, product ion spectra of precursor ions usually provide enough information to unambiguously determine the target compound, but as Humalog and human insulin have identical molecular weights and structures differing only by a change of position of two adjacent amino acids, detailed mass spectral information is of utmost importance. The fragment ion at *m/z* 226 in Figure 3 is composed by proline-lysine (y_3-y_1) originating from the C-terminus of the B-chain, in particular position B_{28}-B_{29}, which is modified in all synthetic insulins investigated in the present study.

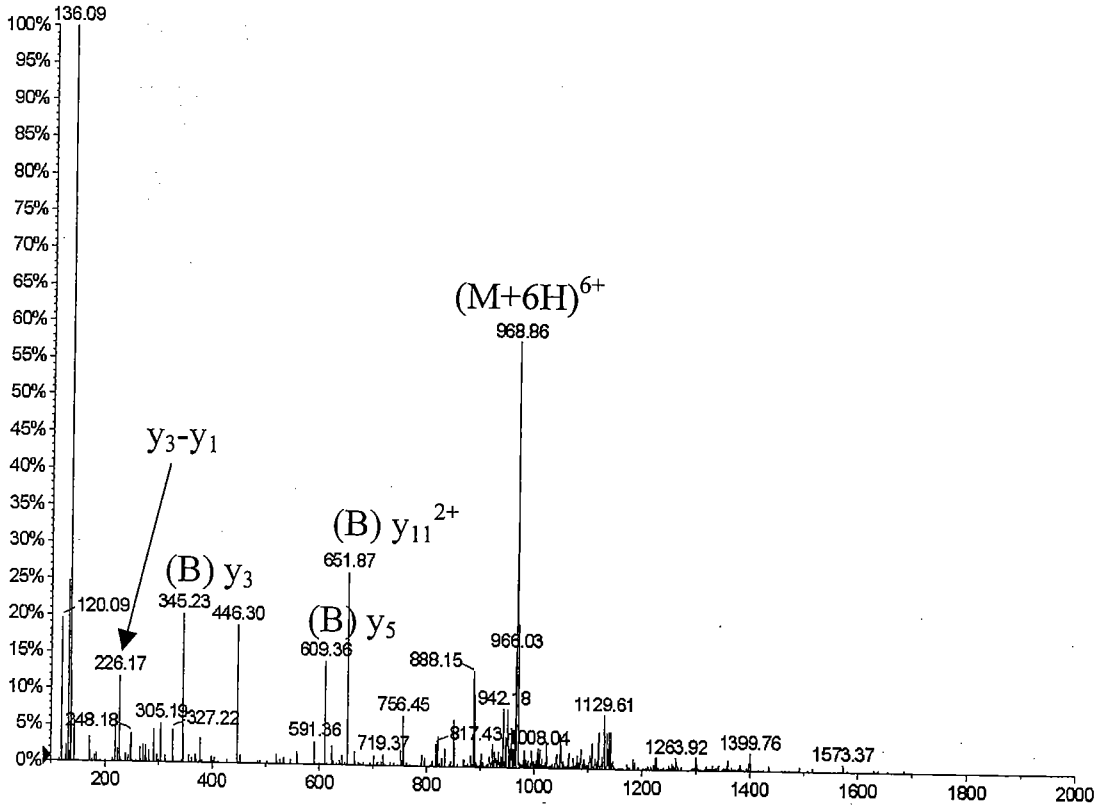

Figure 3: ESI product ion spectrum of *m/z* 968.9 of human insulin. The fragment ion at *m/z* 226 (y_3-y_1) indicates the proline-lysine constitution at B_{28}-B_{29}.

In order to intensify the abundance of this informative fragment ion, cleavage of disulfide bonds was conducted, followed by MS/MS experiments on the resulting B-chains comprising the modifications of interest. In Figure 4, the product ion spectrum of the quadruply charged molecule at *m/z* 858.4 is presented, containing a base peak product ion at *m/z* 226.

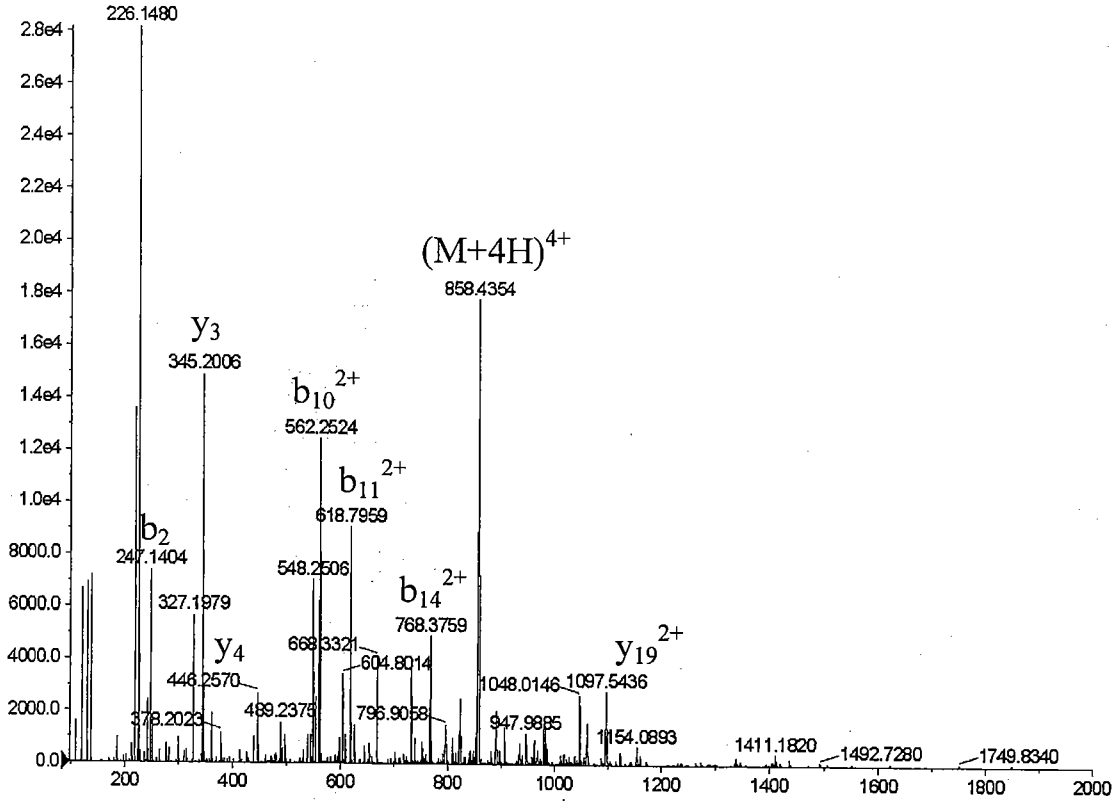

Figure 4: ESI product ion spectrum of *m/z* 858.4 of the B-chain of human insulin. The fragment ion at *m/z* 226 (y_3-y_1) represents the base peak of the spectrum.

The same experiment was performed with Humalog, the corresponding product ion spectrum of which is shown in Figure 5. Here, *m/z* 226 is not visible, but an intense fragment at *m/z* 217 was obtained, composed by the B-chain C-terminal proline-threonine (y_2), which can not be generated from human insulin. Hence, plasma samples containing either Humalog, human insulin, or both, can be analyzed regarding the presence of the synthetic insulin by MS/MS experiments and identification of the characteristic fragment ions at either *m/z* 226 or *m/z* 217. In Figure 6, the chromatogram of a plasma sample fortified with 10 pmol/mL of Humalog and prepared by extraction and disulfide reduction is shown, demonstrating the presence of *m/z* 217 after CAD of the multiply charged B-chain.

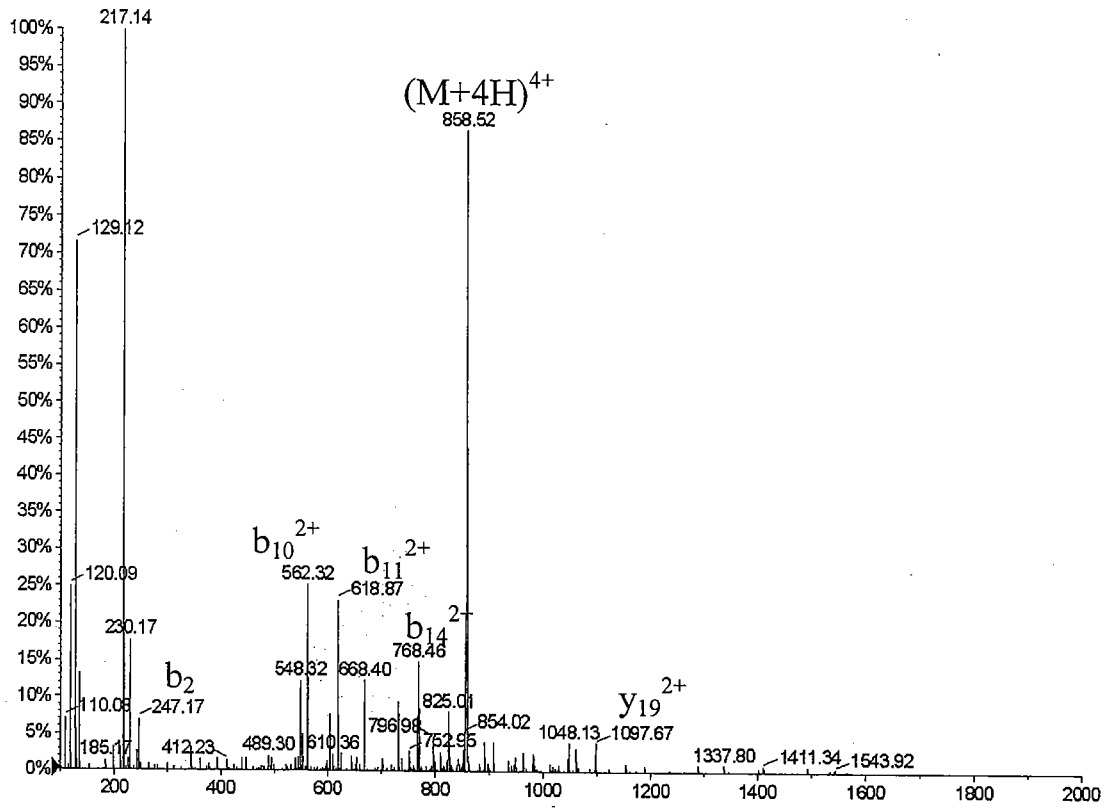

Figure 5: ESI product ion spectrum of *m/z* 858.4 of the B-chain of Humalog. The fragment ion at *m/z* 226 (y_3-y_1) is not present but its counterpart at *m/z* 217 representing the y_2-ion.

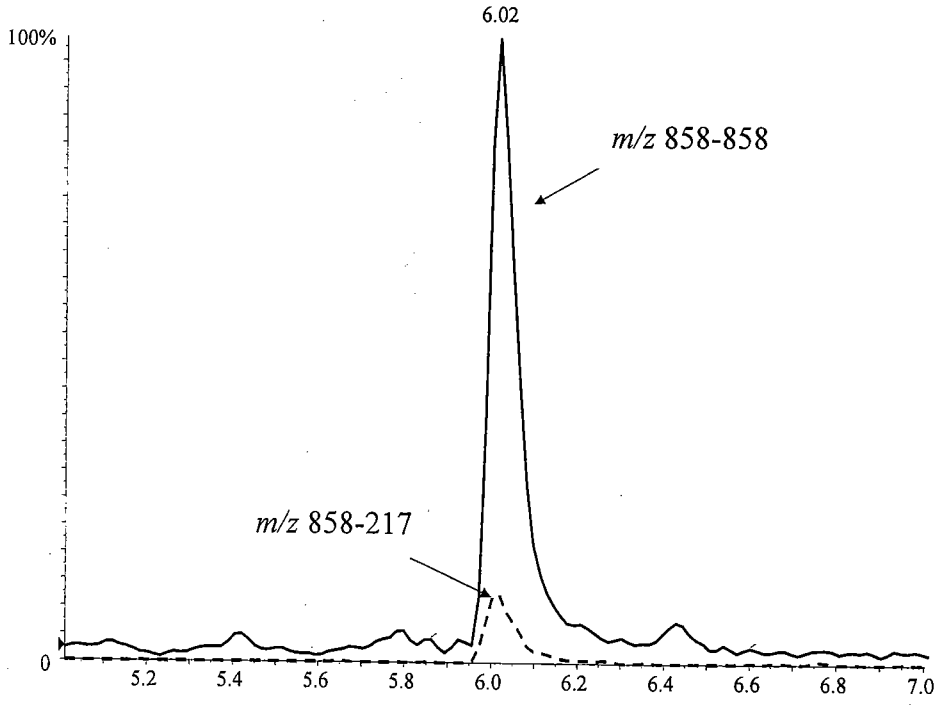

Figure 6: Extracted ion chromatogram of a plasma sample fortified with 10 pmol/mL of Humalog. The presence of *m/z* 217 demonstrates the presence of the synthetic insulin instead of endogenous insulin.

For Novolog and Lantus, the same sample preparation and analytical procedure can be performed providing more detailed information on the analytes than MS/MS of the intact compounds, but here molecular weights differ from endogenous human insulin and thus facilitate their identification compared to the detection of Humalog. Figures 7 and 8 present the product ion spectra of the B-chains of Novolog and Lantus, respectively, after electrospray ionization and CAD.

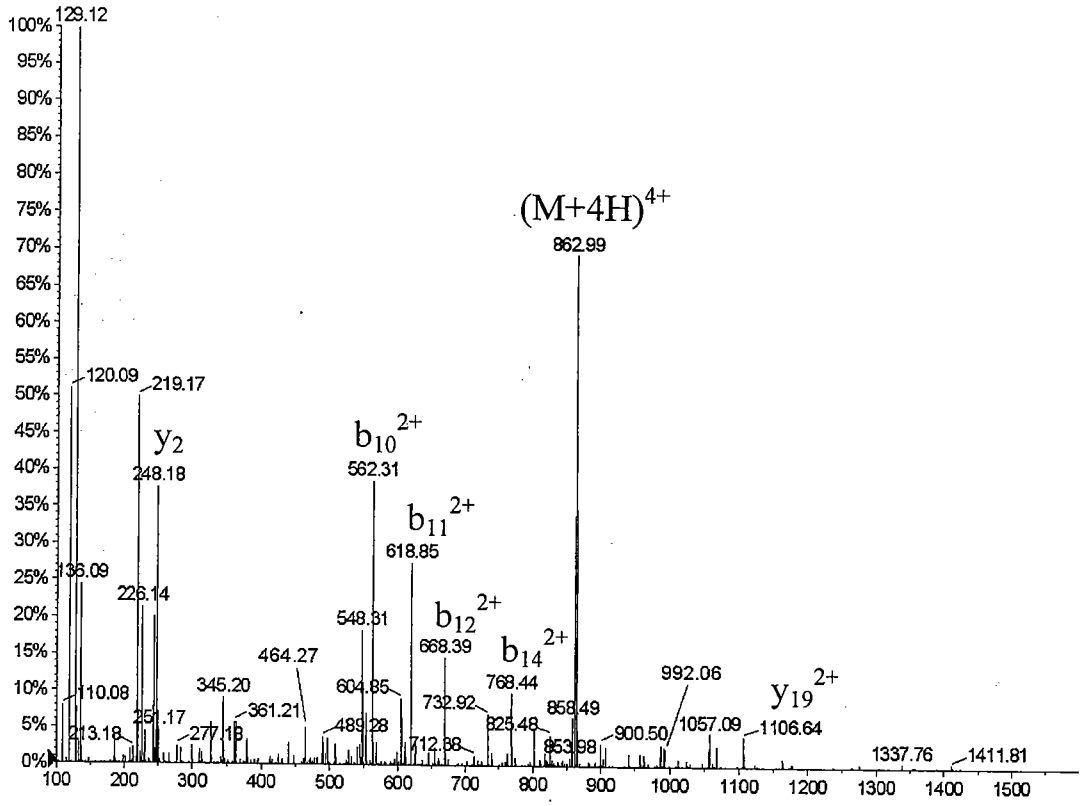

Figure 7: ESI product ion spectrum of *m/z* 862.4 of the B-chain of Novolog

Conclusion

The identification of synthetic insulins in human plasma by LC-ESI-MS/MS is possible by means of CAD of intact and disulfide-reduced analytes. Specific and characteristic fragment ions of synthetic insulins enable their unambiguous determination in a complex matrix such as plasma, but the estimated detection limits of 3 pmol/mL of plasma are not sufficient for doping control purposes and have to be improved by more sophisticated instrumentations, e.g. more sensitive mass spectrometers and/or micro/capillary liquid chromatography.

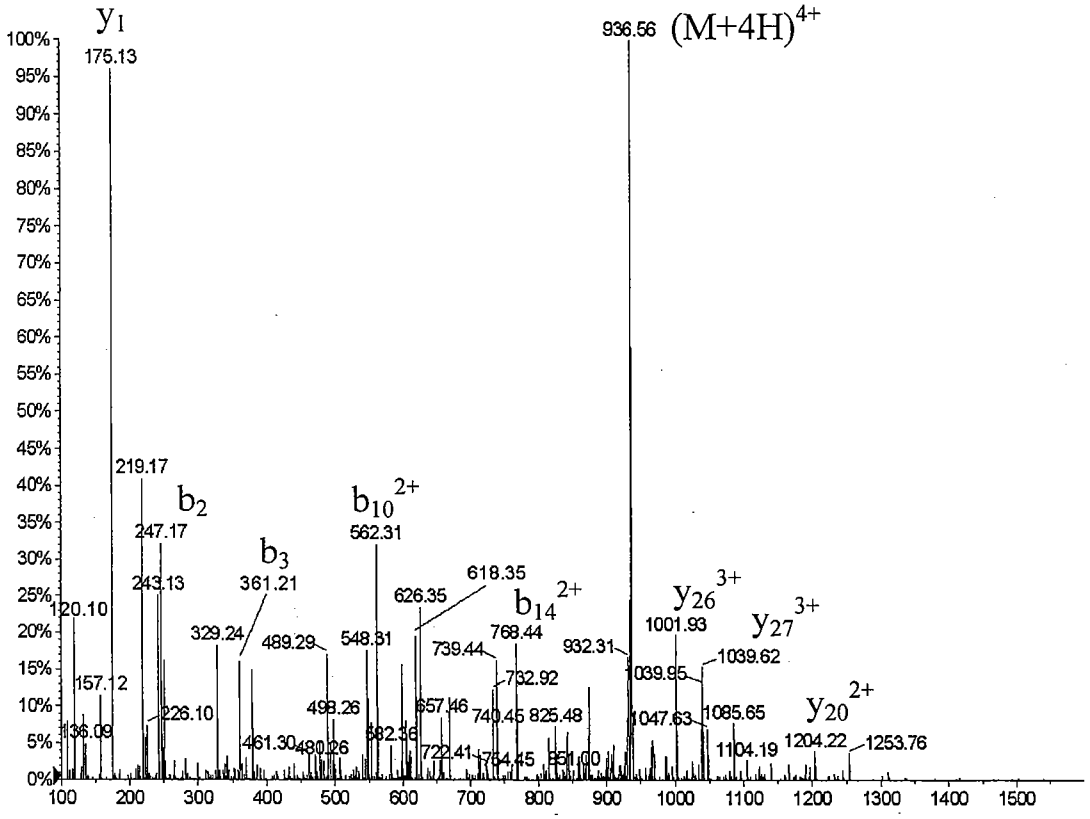

Figure 8: ESI product ion spectrum of *m/z* 936.6 of the B-chain of Lantus

Acknowledgements

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References

[1] International Olympic Committee, List of Prohibited Substances and Methods of Doping. Lausanne, 2003.

[2] Rosak, C. *Internist* **2001**, *42*, 1523-1535.

[3] Fabris, D.; Fenselau, C. *Anal. Chem.* **1999**, *71*, 384-387.

[4] Barnett, A.H.; Owens, D.R. *Lancet* **1997**, *349*, 47-51.

[5] Campbell, K.R.; White, J.R.; Levien T.; Baker, D. *Clin. Ther* **2001**, *12*, 1938-1957.

[6] Levien, T.; Baker, D.E., White, J.R., Campbell, K.R. *Ann. Pharmacother.* **2002**, *36*, 1019-1027.

[7] Reinhardt, L.; Panning, C.A. *Am. J. Health-Syst. Pharm.* **2002**, *59*, 643-649.

[8] Chevenne, D.; Trivin, F.; Porquet, D. *Diabetes Metab.* **1999**, *25*, 459-476.

[9] Yalow, R.S.; Berson, S.A. *Nature* **1959**, *184*, 1648-1649.

[10] Lindström, T.; Hedman, C.A.; Arnqvist, H.J. *Diabetes Care* **2002**, *25*, 1049-1054.

[11] Sapin, R.; Le Galudec, V.; Gasser, F.; Pinget, M.; Grucker, D. *Clin. Chem.* **2001**, *47*, 602-605.

[12] Butter, N.L.; Hattersley, A.T.; Clark, P.M. *Clin. Chim. Acta* **2001**, *310*, 141-150.

[13] Stöcklin, R.; Vu, L.; Vadas, L.; Cerini, F.; Kippen, A.D.; Offord, R.E., Rose, K. *Diabetes* **1997**, *46*, 44-50.

[14] Kippen, A.D.; Cerini, F.; Vadas, L.; Stöcklin, R.; Vu, L.; Offord, R.E., Rose, K. *J. Biol. Chem.* **1997**, *272*, 12513-12522.

[15] Darby, S.M.; Miller, M.L.; Allen, R.O.; LeBeau, M. *J. Anal. Toxicol.* **2001**, *25*, 8-14.