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Determination of insulin to C-peptide ratios as marker for a surreptitious insulin application

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Introduction

The misuse of recombinant human insulin (I) and its chemically modified synthetic analogues were frequently reported in scientific as well as non-scientific literature since more than 10 years.(1, 2) Although the performance enhancing potency is still in discussion, the usage of this prohibited substance seemed to be widespread.

Considering the recent development of mass spectrometry-based approaches to determine the chemically modified synthetic insulin analogues in urine and plasma, an unambiguous assay to uncover the misuse of recombinant human insulin is still missing.(3)

The protein biosynthesis of insulin in the Langerhans cells of the pancreas is characterized by the stepwise enzymatic cleavage of the single chain precursor molecule proinsulin into insulin and C-peptide (C). Thus, the amount of I and C, secreted by exocytosis from the vesicles into the bloodstream, is equimolar and the ratio of I/C in plasma is described to be constant.

Plasma insulin and C-peptide concentrations of 50 healthy volunteers were determined by means of a sensitive ELISA. In addition 20 plasma samples provided from type II diabetics and 6 samples from type I diabetics with known amount of applied insulin were analysed.

Experimental

Fifty plasma samples from healthy volunteers without any current medication and known diseases (male 23, female 27, age 16-35 years) were provided from the department of Cardiology and Sports Medicine of the German Sport University, Cologne, Germany. Twenty

samples from type II diabetic patients (14 male, 6 female, age 62 ± 12 years, height 172 ± 9 cm, weight 97 ± 19 kg) and 6 samples from type I diabetics (2 male, 4 female, age 34 ± 12 years, height 171 ± 3 cm, weight 69 ± 13 kg) were kindly provided from patients being treated in the Diabetes Zentrum Bad Mergentheim (Germany, Prof. Dr. Haak). All samples were stored frozen at –20 °C immediately after collection and thawed prior to analysis in order to avoid degradation.

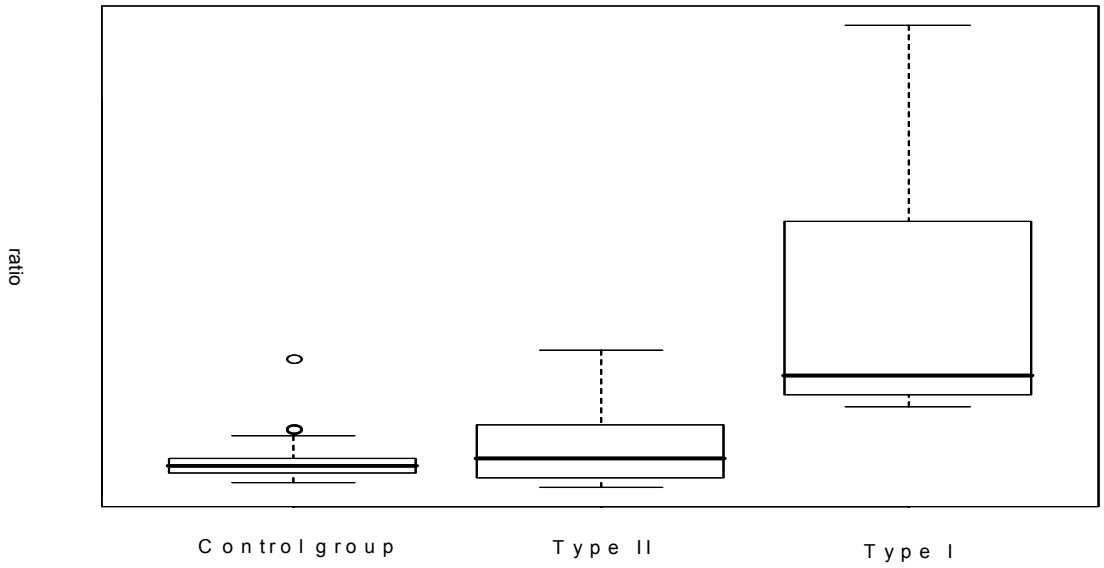

Figure 1: Box plots of I/C-ratios for control group, type II diabetics and type I diabetics.

Insulin and C-peptide assay

Enzyme-Linked Immunosorbent assay (ELISA) kits for insulin and C-peptide determination were purchased from DSL Diagnostic Systems Laboratories, Inc. (Webster, Texas). Performance characteristics for the insulin ELISA (according to the manufacturer's manual) were provided with a limit of detection (LOD) of 0.26 µIU/mL (=0.01 ng/mL), intra-assay precision at three concentration levels, inter-assay precision, recovery rate and linear approximation in the concentration range. Cross reactivity to proinsulin and C-peptide was not detected. Performance characteristics for the C-peptide ELISA (according to the manufacturer's manual) were provided with a LOD (0.012ng/mL), intra-assay precision, inter-assay precision, recovery rates and linear approximation. Sample preparation was performed strictly according to manufacturers instructions in order to guarantee optimal performance for each assay. Quantification was obtained due to a six-point calibration curve

assayed in duplicate covering the whole concentration range for insulin and C-peptide determination, respectively. Additionally, two quality control samples (QC I and II) with known insulin (approx. 0.4 and 1.2 ng/mL) resp. C-peptide (approx. 0.4 and 2.2 ng/mL) concentration were analysed in each set to ensure the accuracy of the assay. Lot specific tolerance rates for QC I and II concentrations were provided from the manufacturer and were fulfilled in each set of analysis.

Results and Discussion

Figures 1 and 2 show the main results of the analysed samples illustrated as box plot (Fig. 1) and bar plot (Fig. 2) of the respective I/C-ratios. Generally, all determined values (control, type I and II group) for insulin and C-peptide were in full compliance to reference values for clinical purposes provided from literature of diabetes care.[4] These reference concentrations of plasma insulin range from 0.05 ng/mL in fasting state and up to 6 ng/mL for non-fasting levels (e.g. after oral glucose tolerance test) for non-obese and non-diabetic volunteers. C-peptide levels range from 0.5 to 8 ng/mL for healthy humans and correlate with the fasting state also. Type II diabetics are known to possess higher values due to a deficiency of the insulin receptor and, thus, an increased endogenous production.[4] In accordance to this the mean values in type II group for I and C are slightly elevated in comparison to the control group. Type I diabetics suffer from an almost complete lack of endogenous I and C production. This leads to very low amounts of C circulating in plasma and the measured I is nearly completely of exogenous origin. High I/C-ratios of type I group were mainly influenced by the very low concentrations of C that are far below physiological concentrations of healthy humans. Therefore, a definition of an I/C-ratio threshold based on data provided from type I diabetics, deliver non physiologic ranges far away from expected values in cheating athletes and provides not a valid tool for doping controls. Plasma half-life, metabolic fate and renal clearance of insulin and C-peptide is known to be exhaustively different due to various biological effects of insulin and almost inert activity of C-peptide in the circulation. Additionally, different storage stability in plasma for both analytes is reported frequently.(4)Insulin is stable at –20°C, 4°C and even at room temperature, while C-peptide is thermally labile even at –20°C. A further aspect is given by the expected amounts of injected exogenous insulin in cheating athletes that is presumably lower than the mean amount in the type II group due to threatening hazard of hypoglycaemic states. The expected influence of even lower amounts of injected insulin on the I/C-ratio is presumably actually less than shown in the type II group where the differences to the control group were not sufficient yet. All

these facts complicate the use of an unambiguous I/C-ratio threshold to uncover the misuse of human insulin. Similar results were also presented by the Australian Sports Drug Testing Laboratory on the 26th Manfred Donike Workshop on Dope Analysis.(5)They conducted an intravenous insulin application on healthy volunteers and measured the I/C-ratio. After a fast increase within a few minutes the ratio was shown to fall back to normal values within about 30 min. An additional oral intake of glucose just prior to insulin application reduces even more the increase of the ratio. These results support our own data and conclusions that were obtained without application to healthy humans or animals due to ethical considerations.

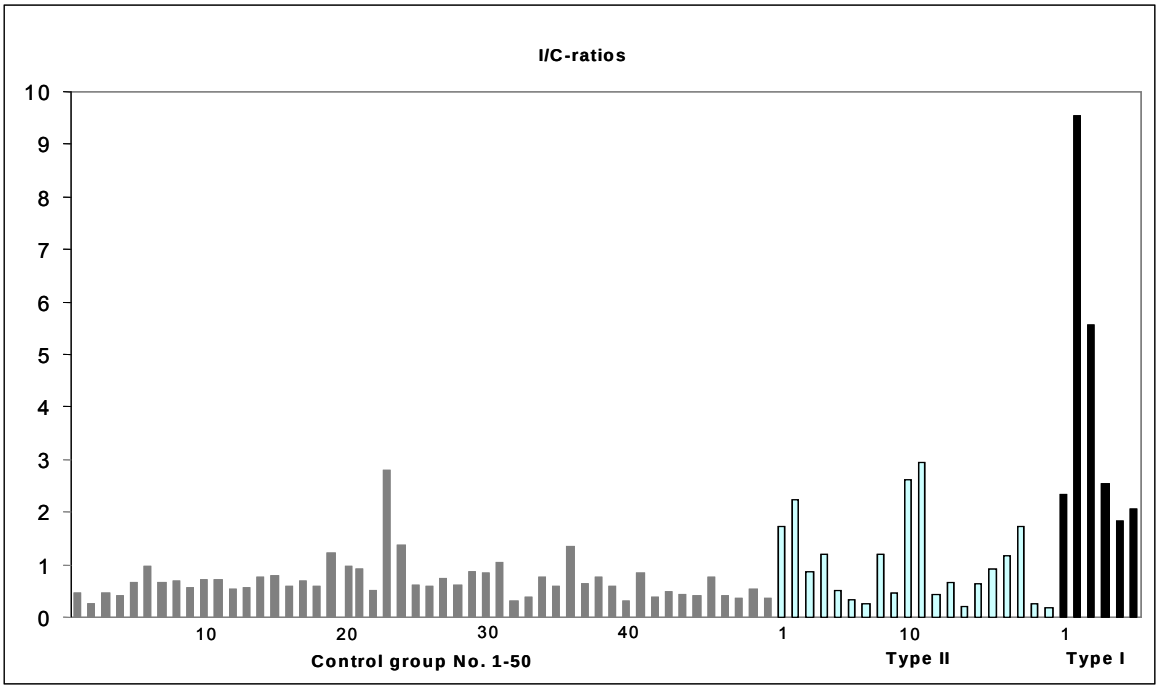

Figure 2: Bar plot of I/C-ratios for control group (grey), type II diabetics (fade grey) and type I diabetics (black).

References

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