Comparison of Zn Content in Rapid-acting Insulin and Biphasic Suspension by FAAS

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Abstract: Various insulin analogs (rapid-acting and intermediate-acting insulin) have been investigated for the determination of zinc content by flame atomic absorption spectrometry. This paper presents the validation of a method and comparison of zinc content in the insulin samples studied. The method was linear ($r^2 = 0.9997$), the limit of detection was 0.0098 mg L$^{-1}$ and the limit of quantification was 0.0296 mg L$^{-1}$, the precision (as relative standard deviation) was up to 7.4 %, and the accuracy was within a range of 95.6 % to 100.1 % for the recovery of fortified insulin samples. The zinc content in the insulin samples ranged from 14.9 mg L$^{-1}$ to 16.3 mg L$^{-1}$ for rapid-acting insulin and 18.7 mg L$^{-1}$ to 19.9 mg L$^{-1}$ for intermediate-acting insulin. A higher zinc content was found in the intermediate-acting insulins than in the rapid-acting insulin analogs ($p < 0.05$). The obtained results should be considered in the establishment of new or improvement of currently available procedures used to assure the quality, safety and efficacy of insulin products.

Keywords: zinc, rapid-acting and intermediate-acting insulins, flame atomic absorption spectrometry.

INTRODUCTION

DIABETES mellitus (DM) is one of the most common chronic endocrine diseases, characterized primarily by a lack of or resistance to insulin, which is involved in glucose metabolism, and remains one of the leading causes of death worldwide.[1–3] Depending on the underlying pathological process, the disease is divided into two main types. Type 1 diabetes, often referred to as juvenile DM, is characterized by the presence of autoantibodies to insulin and to the surface markers of the pancreatic islets, particularly to the β-cells where insulin synthesis occurs. Type 2 diabetes is characterized by the inability of insulin to regulate the entry of glucose into cells, referred to as insulin resistance.[4,5] Human insulin, a polypeptide composed of 51 amino acids, is the main drug used to treat DM in humans, discovered 100 years ago in Toronto 1921 by Banting, Best and Macleod. In a solution of neutral pH, insulin may exist as monomers, dimers, or hexamers, depending on the concentration. In the presence of zinc ions, three dimers associate to form a hexamer in which two zinc ions form a coordination bond with the imidazole group of the B10 histidine residues, the one from each dimer.[6]

Zinc plays a role in the synthesis, storage, and secretion of insulin, as well as in the conformational integrity of insulin in hexameric form. The interaction of zinc with insulin causes conformational changes and enhances binding to the insulin receptor. Insulin hexamers formed in the presence of zinc exhibit greater stability than insulin dimers and monomers.[7,8] The link between zinc and diabetes is not only through insulin, but prolonged uncontrolled diabetes leads to a decrease in total zinc levels in the body through increased urinary excretion, which in turn exacerbates cytokine-induced damage in autoimmune attacks through a mechanism where the immune response is not controlled by zinc. In addition, zinc has an insulin mimetic effect, probably by inhibiting protein tyrosine phosphatase, and also as a cofactor and structural motif ("zinc finger") of important enzymes in glucose metabolism. It is the activator of fructose-1-6-bisphosphate aldolase and the inhibitor of fructose-1-6-bisphosphatase. It may also have antioxidant activity and is a cofactor of copper/zinc superoxide dismutase, the major antioxidant enzyme in the cells.[4,9–12]

The structure of insulin determines the physiological mechanisms of insulin storage and release. By altering the
structure, it is possible to change the pharmacokinetics and pharmacodynamics of insulin, to improve the treatment of diabetes. Insulin analogs have been developed that better mimic the physiological profile of insulin through improved pharmacokinetic properties, resulting in a faster or longer pharmacodynamic effect. The unique pharmacological properties of each insulin analog significantly alter the rate of hexamer dissociation and subsequent movement of free insulin into the circulation, i.e. the time-concentration profile of activity. Thus, the absorption rate of an insulin preparation depends on its self-association state, with monomers being absorbed more rapidly and dimers and hexamers more slowly. With the development of genetic engineering, it has become possible to develop analogues of insulin that do not form zinc-insulin hexamers, resulting in faster uptake at the injection site. Depending on how quickly they begin to act in the human body, insulin preparations are classified as rapid-acting, short-acting, intermediate-acting, and long-acting insulins. One feature of rapid-acting insulins that distinguishes them from other groups of insulin preparations is their ability to mimic normal postprandial insulin secretion. After subcutaneous injection, they rapidly dissociate into active monomeric forms that are rapidly absorbed. They reach their maximum concentration in the blood within one hour of administration reducing the risk of late postprandial hypoglycaemia. Intermediate insulin is a crystalline mixture of regular human insulin and protamine. The mixture is administered as an injection into the subcutaneous tissue from which insulin is released by proteolytic enzymes present in the tissue that degrade protamine, thereby prolonging the time of insulin release from the composition and reducing the number of daily injections. The stability of such insulin formulations is provided by zinc ions.

Many essential elements are involved in myriad metabolic processes in biological systems and often function as structural components of larger molecules and as biocatalysts in various biological reactions. The study of specific elements and their concentrations, distribution, species, or interaction with biomolecules is becoming increasingly important in the emerging field of bioanalysis because of their role in the human organism. Determining the concentration of trace elements, such as zinc, in biological and/or pharmaceutical samples is often challenging due to their low concentrations.

Usually, atomic spectroscopic techniques such as flame atomic absorption spectrometry (FAAS), electrothermal atomic absorption spectrometry (ETAAS), inductively coupled plasma emission spectrometry (ICP-AES), inductively coupled plasma mass spectrometry (ICP-MS) and total reflection X-ray fluorescence spectrometry (TXRF) are used for elemental determination in pharmaceutical samples. In recent years, the number of studies using ICP-MS and ICP-AES has increased due to their multi-element capability, high sample throughput, and high sensitivity. Nevertheless, FAAS is widely used in the pharmaceutical industry and offers some advantages over other spectroscopic techniques, such as a simple workflow with a more cost-effective approach compared to ICP-based techniques and also requires less skill level for an analyst than ICP-AES and ICP-MS.

Due to the physicochemical and physiological relationships between insulin and zinc, the determination of zinc content is one of the important parameters that should be considered in the quality control and assurance of insulin products. The aim of this work was to develop and validate the method of Zn content determination suitable for both rapid-acting and intermediate insulins using FAAS. Furthermore, it was aimed to demonstrate the applicability of developed method on two commercial insulin preparations available in Croatia. The obtained results may contribute to the establishment of new or improvement of currently available procedures used to assure the quality, safety and efficacy of insulin products.

Analytical methods should be used within the framework of Good Manufacturing Practice (GMP) and Good Laboratory Practice (GLP) and must be developed using the protocols and acceptance criteria specified in the International Council for Harmonization (ICH) guidelines Q2(R1). In this paper, typical validation characteristics such as precision, trueness, limits of detection and linearity that should be considered in the validation of any analytical method for the analysis of the content of elements in medicinal products for human use have been evaluated in accordance with the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use. Finally, the results of FAAS analyses of Zn obtained from the rapid-acting insulin samples were compared with those obtained from the intermediate-acting insulin samples.

**EXPERIMENTAL**

**Chemicals and Reagents**

Standard stock solution of Zn (1000 μg mL⁻¹, standard for AAS) was purchased from PerkinElmer (Waltham, MA, USA). Ultrapure deionized water for dilution of stock solution and samples was obtained from a Milli-Q purification system (Millipore Corp., Bedford, Massachusetts). Nitric acid (65 % HNO₃) and hydrochloric acid (36 % HCl) were purchased from Kemika (Zagreb, Croatia). 6 mol L⁻¹ and 0.01 mol L⁻¹ HCl were prepared by diluting 36 % HCl according to the standard procedure. Working standards...
of Zn in the concentration range 0.05 mg L\(^{-1}\) to 1.2 mg L\(^{-1}\) were prepared fresh daily by dissolving the stock solution in 0.01 mol L\(^{-1}\) HCl. All chemicals and solvents used in this study were of analytical reagent grade. All glassware used for standard preparation was soaked in 15 % HNO\(_3\) (p.a. Kemika, Zagreb, Croatia) over-night, and thoroughly rinsed with MilliQ water before use.

**Insulin Samples**

For this study, 6 different batches of each insulin preparation were analysed. The collected samples were kindly provided by Pharmacy Lisjak (Pula, Croatia). Insulin pens with two different onsets of action were used, such as a rapid-acting NovoRapid\(^\text{®}\) Penfil\(^\text{®}\) (RAI) (Novo Nordisk Pharmaceuticals, Denmark; 3 mL, 300 IU mL\(^{-1}\), containing insulin aspart; \(n=6\) batches) and an intermediate-acting NovoMix\(^\text{®}\) 30 FlexPen\(^\text{®}\) (IAI) (Novo Nordisk Pharmaceuticals, Denmark; 3 mL, 300 IU mL\(^{-1}\), containing the solution of insulin aspart/protamine-crystallised insulin aspart in the ratio 30/70; \(n=6\) batches). According to the Croatian Agency for Medicinal Products and Medical Devices, these two products are the most prescribed in Croatia in 2020.\(^{[31]}\)

Insulin samples were prepared according to the procedure described in the monograph European Pharmacopeia.\(^{[32]}\)

3 mL of the sample was transferred to a 100 mL flask and brought to volume with 0.01 mol L\(^{-1}\) HCl. Three replicate measurements were performed for each sample in independent working sessions.

**Instrumentation**

Zn concentration was determined using an AAnalyst 800 atomic absorption spectrometer (Perkin Elmer Instruments, Norwalk, CT, USA) with deuterium background correction under optimized measurement conditions with hollow cathode lamp (PerkinElmer Lumina SingleElement Hollow Cathode Lamp) and at optimum flame height (air-acetylene). The following parameters were used: Zn hollow cathode lamp at 15 mA, wavelength at 213.9 nm, acetylene/air was used as oxidant (pressure/flow: 0.9×10\(^5\)/5; 5.5×10\(^5\)/17 for acetylene and air, respectively). Results were recorded and processed using AAWinlab 32 software (PerkinElmer, Waltham, MA, USA).

**Statistical Analysis**

Statistical analyses were performed using PrismGraphPad 9 (GraphPad Software, Inc., San Diego, USA) and XLStat (Addinsoft Inc, New York, USA). Measurements were performed in triplicate and results were expressed as mean ± standard deviation. The Mann-Whitney U test was used to test hypotheses about differences between Zn content in the insulin samples studied. All conclusions in this work were implemented with a significance level of \(p < 0.05\).

**RESULTS AND DISCUSSION**

In order to determine the zinc content in different insulin preparations (RAI and IAI), the FAAS method used was validated according to ICH\(^{[33]}\) (see Table 1, for details). Typical validation characteristics that should be considered according to this guideline include the following parameters: Linearity, limit of detection (LOD) and limit of quantification (LOQ), trueness and precision ( repeatability and medium precision). The above validation parameters were evaluated to test the real applicability of the methodology for the intended purpose.

**Validation of the Method**

**LINEARITY**

The linearity of the method was determined by constructing a calibration curve from zinc working solutions with concentrations ranging from 0.05 mg L\(^{-1}\) to 1.2 mg L\(^{-1}\) (Figure 1). The obtained calibration parameters are presented in Table 2. As shown, the regression coefficient was determined from the linear regression curve using the absorbance and concentration of the standards with a value greater than 0.999, indicating good linearity over the range studied. In addition, the relative standard deviation (RSD) values obtained from the analysis of triplicate metal standards at the extremes of the indicated range were also acceptable.

**PRECISION**

Precision was assessed by repeatability and intermediate precision studies. Precision studies were performed by analysing spiked insulin samples at the level of 0.2 mg L\(^{-1}\). Repeatability was evaluated using six consistently repeated measurements from one sample and calculation of relative standard deviation (RSD) (Table 3). All samples were prepared and measured under the same experimental conditions. Samples were prepared containing the element of interest at concentrations of 0.4 mg L\(^{-1}\), 0.8 mg L\(^{-1}\), and 1.2 mg L\(^{-1}\). The obtained calibration parameters are presented in Table 2. As shown, the regression coefficient was determined from the linear regression curve using the absorbance and concentration of the standards with a value greater than 0.999, indicating good linearity over the range studied. In addition, the relative standard deviation (RSD) values obtained from the analysis of triplicate metal standards at the extremes of the indicated range were also acceptable.

Table 1. Analytical parameters to be considered and acceptance criteria according to the ICH

<table>
<thead>
<tr>
<th>Analytical parameter</th>
<th>Evaluation</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity</td>
<td>6 standard solutions of Zn up to 1.2 mg L(^{-1})</td>
<td>(r^2 &gt; 0.999)</td>
</tr>
<tr>
<td>Precision</td>
<td>3 independent replicate preparations at the level of 0.1 mg L(^{-1}), 0.2 mg L(^{-1}) and 0.4 mg L(^{-1})</td>
<td>RSD ≤ 5%</td>
</tr>
<tr>
<td>Accuracy</td>
<td>3 independent replicate preparations at the level of 0.1 mg L(^{-1}), 0.2 mg L(^{-1}) and 0.4 mg L(^{-1})</td>
<td>Recovery: 80 – 120%</td>
</tr>
</tbody>
</table>
1.2 mg L⁻¹, and two different insulin samples; rapid-acting insulin (RAI) and intermediate-acting insulin (IAI) spiked at level 0.2 mg L⁻¹. As can be seen in Table 3, the uncertainty obtained is related to the instrument and the counting statistics, and it was found that the RSD values for all samples were below 3 %. To investigate the overall precision of the proposed method, six independent of the fortified insulin samples were prepared and measured under the same experimental conditions. The calculated RSD results are shown in Table 3. A difference in precision was observed for the two targets studied. It was found that the precision for the RAI samples was 5.06 %, while the precision for the IAI samples was 7.37 %. It can be concluded that the experimental procedure is of good quality considering that the values obtained by this procedure take into account the uncertainty due to sample preparation, instrument and counting statistics.

Moreover, from the obtained data and considering the variance models \( \sigma^2_{\text{total}} = \sigma^2_{\text{sample preparation}} + \sigma^2_{\text{FAAS measurement}} \) (1), it can be assumed that the total variance is given by the results of the six individual insulin samples prepared, since it is subject to the influence of both sample preparation (six different samples) and FAAS measurements (six separate measurements of the same sample). In contrast, the results of a single sample exclude the influence of sample preparation. Using the values for RSD presented in Table 3, it can be calculated that the influence of sample preparation is less than 7.37 %.

Intermediate precision was evaluated using the same sample (previously used in the repeatability study) of insulin under test, analysed over a period of three different days (Table 4). The RSD values obtained were less than 5 %. These data indicate a high precision of the method.

**ACCURACY**

Accuracy was evaluated by analysing two independent insulin samples (0.5 mL sample of insulin is diluted to 50 mL with 0.01 mol L⁻¹ HCl) spiked with a reference solution (10 mg L⁻¹) at concentrations of 0.1 mg L⁻¹, 0.2 mg L⁻¹, and 0.4 mg L⁻¹ and calculating recovery values (three replicates were analysed for each concentration). The results obtained are presented in Tables 5. The Zn added to the samples was quantified with recoveries higher than 95 %. Moreover, recoveries between 95.6 % and 100.1 % were obtained for all spiked values. Thus, acceptable recoveries were obtained for all spiked values for both insulin samples studied, confirming the accuracy of the procedure and the absence of matrix effects for this type of samples.

**LIMITS OF DETECTION AND QUANTIFICATION**

The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the expression displayed below. As shown, in both cases the intercept and \( S_y/x \) values of the regression lines obtained from the analysis of a set of calibration standards were used to estimate LOD and

![Figure 1. The calibration curve obtained from Zn standards in concentration range 0.05–1.2 mg L⁻¹ (each point represents the average of the three measurements).](image)

<table>
<thead>
<tr>
<th>Element</th>
<th>Range (mg L⁻¹)</th>
<th>( r^2 )</th>
<th>( a \pm S_a )</th>
<th>( b \pm S_b )</th>
<th>RSD (%)(^{(a)} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn</td>
<td>0.05 – 1.2</td>
<td>0.9997</td>
<td>0.0015 ± 0.0005</td>
<td>0.1536 ± 0.0008</td>
<td>1.4 (0.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.8 (0.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.95 (0.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.1 (0.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.6 (0.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05 (0.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.25 (1.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.9 (1.2)</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Values in parentheses are the Zn concentrations (mg L⁻¹) for which the RSD was calculated (triplicate analysis).

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LOD:
\[ \text{LOD} = y_a + 3s_a \]  
LOQ:
\[ \text{LOQ} = y_a + 10s_a \]  
where \( y_a \) is the intercept and \( s_a \) is the slope of the regression line.

From these equations, LOD and LOQ were calculated using 3 mL of sample and a final volume of 100 mL and were 0.0098 mg L\(^{-1}\) and 0.0296 mg L\(^{-1}\), respectively. In both cases, suitable values for regulatory requirements were obtained. Moreover, the LOD and LOQ obtained in this work are comparable to the values reported in the literature for the determination of Zn in different insulin preparations using atomic spectroscopy techniques. \([34-36]\)

**Application of the Methodology to Determine Content of Zn in the Insulin Samples**

The validated analytical method was applied to the analysis of Zn in two different insulin samples used for the treatment of diabetes mellitus (six different batches of the target insulin sample), namely rapid-acting NovoRapid\(^{\text{®}}\) Penfill\(^{\text{®}}\) (RAI) and biphasic insulin suspension, a mixture of rapid-acting and longer-acting insulin (intermediate-acting NovoMix\(^{\text{®}}\) 30 FlexPen\(^{\text{®}}\), IAI). Insulin samples were prepared according to the procedure described above in the text. The results obtained for the six different batches investigated are shown in Figure 2. For all batches analysed, good agreement was obtained between the Zn concentrations determined in both samples.

**Table 3.** Precision study for Zn determination at 3 different concentration levels (0.4 mg L\(^{-1}\), 0.8 mg L\(^{-1}\), and 1.2 mg L\(^{-1}\)) and measuring a spiked insulin sample at the level 0.2 mg L\(^{-1}\) of Zn in two target samples (RAI and IAI).

<table>
<thead>
<tr>
<th>Nominal Zn concentration (mg L(^{-1}))</th>
<th>0.4</th>
<th>0.8</th>
<th>1.2</th>
<th>RAI(^{\text{®}}) 1 sample / 6 measurements</th>
<th>IAI(^{\text{®}}) 1 sample / 6 measurements</th>
<th>RAI(^{\text{®}}) 6 samples / 1 measurement</th>
<th>IAI(^{\text{®}}) 6 samples / 1 measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>measured Zn concentration (mg L(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td>(0.357 spiked sample)(^{\text{ii}})</td>
<td>(0.385 spiked sample)(^{\text{ii}})</td>
<td>(0.357 spiked sample)(^{\text{ii}})</td>
<td>(0.385 spiked sample)(^{\text{ii}})</td>
</tr>
<tr>
<td>0.413</td>
<td>0.802</td>
<td>1.231</td>
<td>0.353</td>
<td>0.381</td>
<td>0.352</td>
<td>0.455</td>
<td></td>
</tr>
<tr>
<td>0.394</td>
<td>0.807</td>
<td>1.195</td>
<td>0.360</td>
<td>0.393</td>
<td>0.382</td>
<td>0.435</td>
<td></td>
</tr>
<tr>
<td>0.400</td>
<td>0.801</td>
<td>1.193</td>
<td>0.352</td>
<td>0.382</td>
<td>0.372</td>
<td>0.393</td>
<td></td>
</tr>
<tr>
<td>average (mg L(^{-1}))</td>
<td>0.402</td>
<td>0.805</td>
<td>1.201</td>
<td>0.352</td>
<td>0.385</td>
<td>0.359</td>
<td>0.412</td>
</tr>
<tr>
<td>SD</td>
<td>0.066</td>
<td>0.004</td>
<td>0.015</td>
<td>0.005</td>
<td>0.004</td>
<td>0.02</td>
<td>0.003</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>1.64</td>
<td>0.51</td>
<td>1.23</td>
<td>2.52</td>
<td>1.11</td>
<td>5.06</td>
<td>7.37</td>
</tr>
</tbody>
</table>

\(^{\text{ii}}\) rapid acting insulin analog  
\(^{\text{iii}}\) intermediate-acting insulin analog  
\(^{\text{iv}}\) calculated Zn in spike sample = (content of Zn in RAI sample + spike)  
\(^{\text{v}}\) calculated Zn in spike sample = (content of Zn in IAI sample + spike)  
RAI = rapid-acting NovoRapid\(^{\text{®}}\) Penfill\(^{\text{®}}\); IAI = intermediate-acting NovoMix\(^{\text{®}}\) 30 FlexPen\(^{\text{®}}\)

**Table 4.** Intermediate precision study (Inter-day variability) for Zn determination at 3 different concentration levels (0.4 mg L\(^{-1}\), 0.8 mg L\(^{-1}\), and 1.2 mg L\(^{-1}\)) and measuring a spiked insulin sample at the level 0.2 mg L\(^{-1}\) of Zn in two target samples.

<table>
<thead>
<tr>
<th>Nominal Zn concentration (mg L(^{-1}))</th>
<th>0.4</th>
<th>0.8</th>
<th>1.2</th>
<th>RAI(^{\text{®}}) sample (0.357 mg L(^{-1}) spiked sample)(^{\text{vi}})</th>
<th>IAI(^{\text{®}}) sample (0.385 mg L(^{-1}) spiked sample)(^{\text{vi}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>measured Zn concentration (mg L(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td>(0.357 mg L(^{-1}) spiked sample)(^{\text{vi}})</td>
<td>(0.385 mg L(^{-1}) spiked sample)(^{\text{vi}})</td>
</tr>
<tr>
<td>1st day</td>
<td>0.401</td>
<td>0.8055</td>
<td>1.201</td>
<td>0.352</td>
<td>0.385</td>
</tr>
<tr>
<td>2nd day</td>
<td>0.393</td>
<td>0.7665</td>
<td>1.233</td>
<td>0.337</td>
<td>0.432</td>
</tr>
<tr>
<td>3rd day</td>
<td>0.433</td>
<td>0.837</td>
<td>1.158</td>
<td>0.379</td>
<td>0.455</td>
</tr>
<tr>
<td>average (mg L(^{-1}))</td>
<td>0.409</td>
<td>0.803</td>
<td>1.197</td>
<td>0.356</td>
<td>0.424</td>
</tr>
<tr>
<td>SD</td>
<td>0.021</td>
<td>0.035</td>
<td>0.037</td>
<td>0.009</td>
<td>0.006</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>5.20</td>
<td>4.38</td>
<td>3.12</td>
<td>2.54</td>
<td>1.42</td>
</tr>
</tbody>
</table>

\(^{\text{vi}}\) rapid acting insulin analog  
\(^{\text{vii}}\) intermediate-acting insulin analog  
\(^{\text{viii}}\) calculated Zn in spike sample = (content of Zn in RAI sample + spike)  
\(^{\text{ix}}\) calculated Zn in spike sample = (content of Zn in IAI sample + spike)  
RAI = rapid-acting NovoRapid\(^{\text{®}}\) Penfill\(^{\text{®}}\); IAI = intermediate-acting NovoMix\(^{\text{®}}\) 30 FlexPen\(^{\text{®}}\)

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According to the data obtained, as can been seen in Figure 2, a higher concentration of zinc was found in the biphasic insulin mixture than in the rapid-acting insulin. Using the Mann-Whitney U test, there is a statistically significant difference (15.72 ± 0.014 mg L⁻¹ vs. 19.28 ± 0.015 mg L⁻¹; p < 0.05) between the insulin preparations tested, based on the zinc content measured. It is well known that for any insulin analog, the specific changes to the insulin molecule are relatively small, involving only one or two amino acid changes. These changes attenuate the tendency for self-association in hexamers, allowing for more rapid absorption. As mentioned earlier, Zn, which acts as a stabilizer and is present in all insulin preparations, has effects on the physical stability of insulin, including unfolding and non-native aggregation. In the presence of excess zinc, more than 75% of insulin is in the hexameric state and the hexamer becomes the dominant species with greater stability than insulin dimers and monomers. Some previous work has shown that the addition of zinc to insulin should facilitate the formation of hexamers and increase insulin stability. Based on these findings, small amounts of Zn are still added to some long-acting insulin analogs in some cases. The lower stability of insulin hexamers was achieved by rational exchange at the surface of the monomer-monomer interaction and removal of zinc-binding sites. This resulted in faster dissociation after the application of insulin.

In addition, all insulin molecules are expected to be in the form of hexamers and thus have a greater stabilizing effect by further delaying absorption. Based on these findings, the higher Zn content found in the IAI samples is in line with expectations, as the biphasic insulin suspension is a mixture of rapid-acting and intermediate-acting insulin whose prolonged action is due to the zinc-forming complex. The values reported in this study for Zn content in insulin analogues are slightly lower than those reported in previously published work. A possible reason for this could be a different type of insulin analogue analysed or a sample produced by different manufacturer.

**CONCLUSIONS**

FAAS was demonstrated to be a simple, accurate and precise analytical method for the determination of zinc content in two different insulin analogs. The method fulfilled basic requirements of validation according to the ICH guidelines Q2 (R1). The study also showed that there are significant differences in the Zn content of the insulin samples studied. Higher zinc content was found in intermediate acting insulin compared to rapid acting insulin. The obtained results should be considered in the establishment of new or improvement of currently available procedures used to assure the quality, safety and efficacy of insulin products.

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