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Application of scanning electron microscopy and energy-dispersive X-ray analysis to the solution behaviour of Zn–insulin: precipitation phenomena

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Abstract: Scanning electron microscopy (SEM) has been applied in combination with energy dispersive X-ray analysis (EDAX) to identify and analyse particles or particulate matter, occasionally present in clear neutral Zn–insulin solutions. SEM photographs revealed the existence of three different types of precipitate, consisting of particles with a crystalline, amorphous or gel-like nature, respectively. At present, it is not clear which conditions lead specifically to each of these three types of precipitate. The advantages of the EDAX method are shown. The technique enables semi-quantitative analysis to be performed on a single particle as small as 0.2 μm. It was demonstrated with the EDAX method that the particles occasionally found in clear Zn–insulin solutions contain insulin as well as Zn in roughly the same ratio as in the insulin starting material. It is concluded that the EDAX method has great potential in pharmaceutical technology, *inter alia* for the analysis of emulsion systems (in the frozen state), as well as suspensions and particulate matter in injection fluids. This technique is particularly useful in the latter case, due to its applicability to extremely small sample sizes.

Keywords: *Scanning electron microscopy (SEM); energy dispersive X-ray analysis (EDAX); insulin; complexation; precipitation.*

Introduction

This paper describes the application of scanning electron microscopy (SEM) in conjunction with energy dispersive X-ray analysis (EDAX) to identify presumed insulin precipitates in clear Zn–insulin solutions. With the apparatus employed, the presence of elements with an atomic number greater than 9 can be readily demonstrated. Moreover, these elements can be semi-quantitatively analysed, irrespective of the nature of the forces which bind them to protein (e.g. ionic binding, chelation).

According to the European and British Pharmacopoeias, traces of precipitated insulin are allowed in insulin solutions for injection [1, 2]. The mechanism of insulin

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precipitation as well as the precipitates formed have been the subject of a number of investigations [3, 4]. Among the compounds known to induce precipitation of insulin are amines (e.g. protamine) and cations such as Zn^{2+}, Ca^{2+}, Cu^{2+} and Co^{3+} [3–5].

In addition, insulin precipitation is influenced by ionic strength of the solution, temperature, pH, degree of purity and proinsulin content. Finally, factors such as fluid motion and the shear stress applied also play a role in the precipitation process [4, 6].

In many cases, interactions of cations with insulin have been studied by physico-chemical examination of crystalline complexes, or by monitoring complex formation and, if applicable, precipitation directly in, or from, the dissolved state. To a much lesser extent, microchemical techniques have been employed to analyse the precipitates resulting from such complex formation.

Materials and Methods

Preparation of the Zn–*insulin solution*

Bovine insulin was chromatographically purified and subsequently crystallized as Zn–insulin. The Zn content of the crystals was determined by atomic absorption spectrometry to be 0.41% (m/m) Zn. This insulin was dissolved in an aqueous solution of methyl *p*-hydroxybenzoate, after which sodium acetate and sodium chloride were added. Subsequently, the pH was adjusted to 7.35 and the solution was sterilized by 0.2-μm filtration. Finally, the solution was poured into vials.

The insulin solution had the following composition:

— chromatographically purified bovine insulin (CPI), 1.6 mg ml^{-1};
— methyl *p*-hydroxybenzoate, 1.0 mg ml^{-1};
— sodium acetate (calculated as sodium acetate·$3H_2O$), 1.36 mg ml^{-1};
— sodium chloride, 7.0 mg ml^{-1}.

In such solutions, precipitates were formed occasionally upon prolonged storage at 4–8°C or room temperature.

Isolation of precipitates for investigation by SEM and EDAX

The precipitates present in the Zn–insulin solution were collected on a Nuclepore filter (polycarbonate 0.2 μm, Nuclepore Corporation). Subsequently, the Nuclepore filters were mounted on specimen holders with a surface of pure carbon.

For analysis by EDAX and imaging with "material contrast" the samples were coated with a thin layer of carbon by evaporation.

For optimum imaging of the particles the specimens were, after performing EDAX, sputter-coated with Au.

SEM and EDAX analysis

A Cambridge S180 SEM with conventional secondary-electron imaging was used. The apparatus was equipped with an energy dispersive X-ray analyser (EDAX 711) and an annular semi-conductor system (ESO) for the detection of high-energy X-rays reflected by about 180°. By operating the detectors synchronously information can be obtained about: (a) the morphological features of the precipitate by means of secondary electron imaging; (b) the nature and relative abundance of the inorganic elements by EDAX; and (c) the distribution of heavy elements and/or areas of high density, via imaging in the material contrast mode with reflected electrons.

Results and Discussion

Scanning electron microscopy (SEM)

The precipitates consisted of particles with a crystalline, amorphous or gel-like nature. At present, it is unclear why preparation of some solutions resulted in the precipitation of crystalline particles, whereas preparation of other solutions with the same composition yielded not crystalline, but amorphous precipitates. SEM photographs of the crystalline and amorphous precipitates, respectively, are given in Figs 1(a) and (b). Both precipitates consist of particles varying in size between about 0.5–5 μm (maximum diameter). The individual particles can be very clearly observed in Fig. 1. The EDAX method is capable of determining the composition of each individual particle. The EDAX analyses are described below.

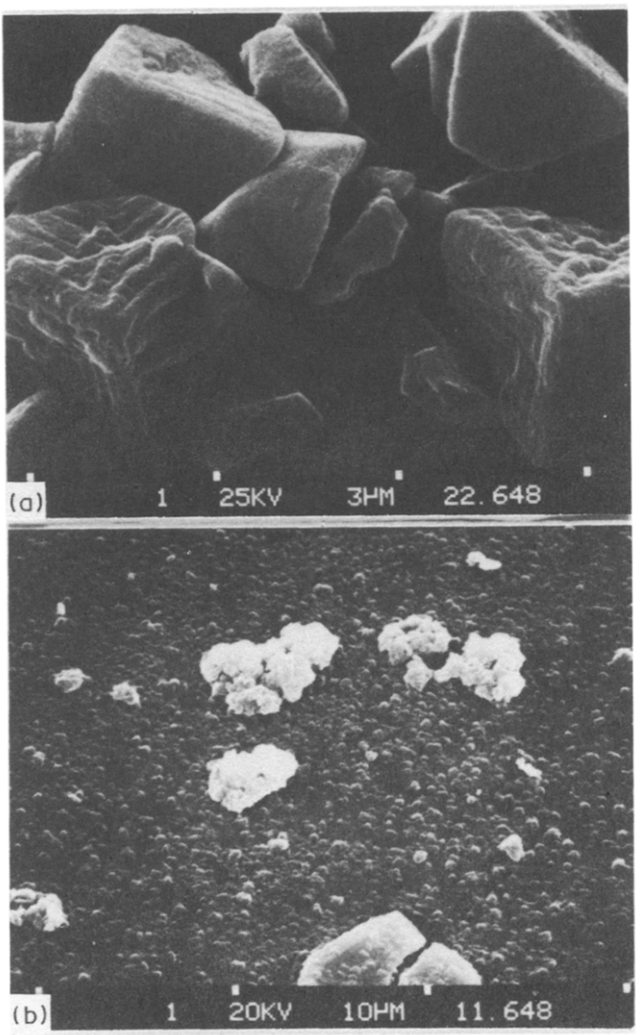

Figure 1
SEM photographs of the crystalline (a) and amorphous (b) Zn–insulin precipitates.

EDAX analysis of insulin raw material

Sulphur. Figure 2(a) shows an EDAX spectrum of CPI. For this analysis a single Zn–insulin crystal has been used. The EDAX spectrum of Fig. 2(a) is representative for this type of Zn–insulin, because identical EDAX spectra could be recorded on 10 different Zn–insulin crystals varying in size. The strong signal at 2.3 keV has been assigned to the sulphur atoms present in the insulin molecule (Table 1). In total, six

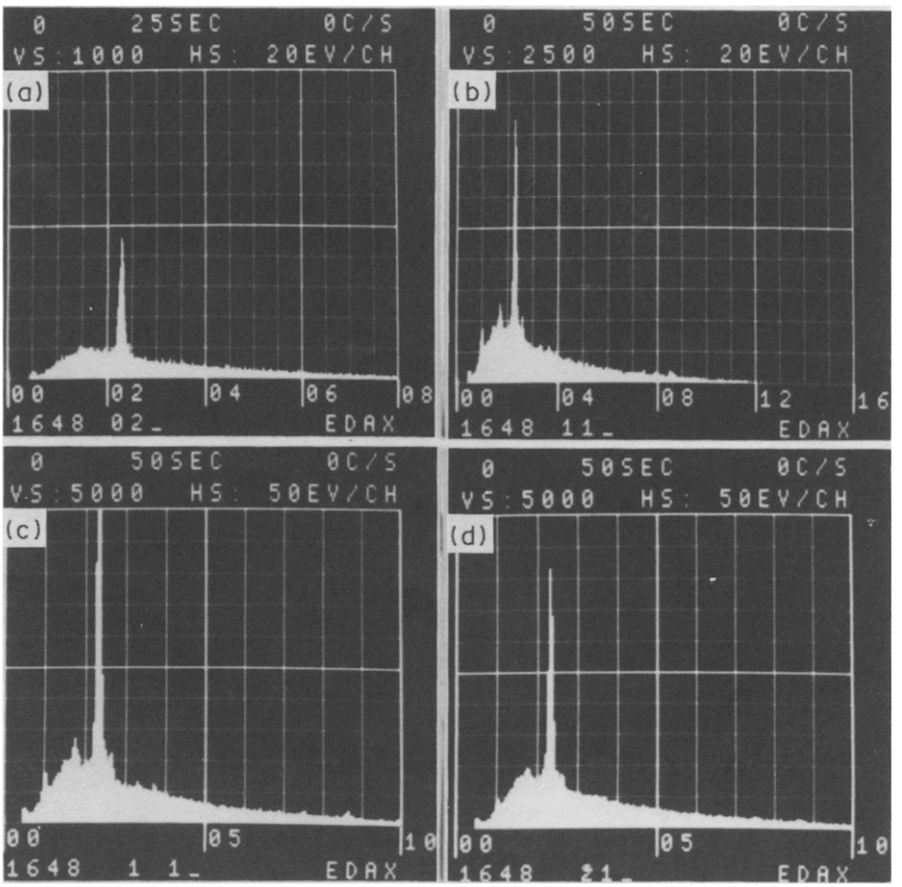

Figure 2. (a) EDAX spectrum of CPI, which shows the strong signal at 2.3 keV, originating from sulphur. (b) EDAX spectrum of the amorphous Zn–insulin precipitate. (c) EDAX spectrum of the gel-like Zn–insulin precipitate. (d) EDAX spectrum of the crystalline Zn–insulin precipitate.

Table 1
Energies (in keV) of the various elements in EDAX spectra

Element	Energy (keV)
Na, Zn (L_α)	1.0
S	2.3
Ca	3.7
Zn (K_α)	8.6

sulphur atoms per insulin monomer [7] contribute to the X-ray intensity at 2.3 keV, and this number of sulphur atoms corresponds to 3.2% S (m/m). The sulphur atoms are present as disulphide bridges between three pairs of cysteinyl residues. EDAX spectra as presented in Fig. 2, made under well-defined conditions on small, pure particles of insulin, can be considered as reference spectra. The S signals can be compared with those of the precipitates, and the S intensity can be used as an "internal standard" for the presence and amount of insulin in and/or on precipitated particles (see below).

EDAX analysis of precipitates

Figures 2(b)–(d) show representative EDAX spectra of the precipitates. Generally, amorphous, crystalline and gel-like precipitates yielded similar EDAX spectra (Figs 2(b)–(d)). Each of the EDAX spectra was recorded using a single particle of the precipitate.

In addition to the strong signal at 2.3 keV assigned to sulphur other, weaker, signals are present in Figs 2(b)–(d). In Table 1, the assignments of these signals to specific elements are given. Three of these are discussed in more detail below, i.e. zinc, sodium and calcium. The history of the signal at 1.8 keV, which is not present in the CPI standard, is unclear.

Zinc. The weak signal observed at 8.6 keV is assigned to Zn. The amount of Zn incorporated into CPI was found by atomic absorption spectrometry to be 0.41%, corresponding to two atoms of Zn per insulin hexamer. The area from which the X-ray signals are excited is not the same for Zn and S due to the (probably) inhomogeneous distribution of Zn over the surface and the interior of the particle. Although the optimum measuring conditions for Zn differ from those of S, nevertheless a comparison of spectra of the three types of precipitated particles, measured under equal conditions, gives approximately equal Zn/S intensity ratios. Because the sulphur intensity depends on the amount of insulin which contributes to the X-ray spectrum, the concentration of Zn in the three types of insulin precipitate is thus shown to be approximately the same.

Sodium. In principle, both Na and Zn can contribute to the signal intensity at 1.0 keV (see Table 1). However, this signal is not present in the Zn-containing CP standard, and consequently, it can be assigned to Na (see also "Materials and Methods"), which is present in the composition.

Calcium. In Figs 2(b) and (d) (amorphous and gel-like particles) a weak signal is present at 3.7 keV. This signal can be assigned to Ca^{2+}. It is well known [3] that cations like Ca^{2+} can replace Zn^{2+} in their capacity to bind to insulin. In Fig. 2(d) (crystalline particles) the Ca^{2+} signal is not detectable, which may point to a preferential affinity of Ca^{2+} for the amorphous and gel-like insulin. The EDAX technique is unable to distinguish whether Ca^{2+} is uniformly bound or may have acted as a nucleation centre for the insulin to precipitate, in which case it will be non-uniformly distributed [8, 9].

Conclusions

In order to identify and analyse particles or particulate matter, occasionally present in clear neutral Zn–insulin solutions, the SEM and EDAX methods were used in combination. With SEM photographs the existence of three different types of precipitate

was demonstrated. These precipitates consisted of particles with a crystalline, amorphous or gel-like nature, respectively.

The EDAX technique is particularly useful for these precipitates, because single particles as small as 0.2 μm can be analysed. The particles were found to be composed of insulin and Zn in roughly the same ratio as in CPI. The precipitation of insulin is a statistical process, in which the lag time for nucleation can vary considerably. Furthermore, the EDAX technique can demonstrate the presence of other elements. It is to be expected that EDAX will find more applications in the field of biochemistry and pharmacy in future due to its powerful resolution and capability for multi-element analyses at an (extremely) small sample size.

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