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Aggregation of insulin by chlorpromazine

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Abstract

Chlorpromazine (CPZ) is known to induce hyperglycaemia and can inhibit insulin secretion in both normal subjects and patients with latent diabetes mellitus. In this study, we have tried to determine a probable mechanism by which CPZ causes hyperglycaemia. It is possible that CPZ causes insulin aggregation by the reduction of disulphide bonds, thereby inactivating insulin and hence causing hyperglycaemia.

Keywords: Chlorpromazine; Insulin; Dithiothreitol

1. Introduction

CPZ (molecular weight 315) is an antipsychotic agent (Fig. 1) widely used by psychiatrists all over the world for the treatment of psychoses [1]. It possesses a tricyclic ring with a 139.4 Å bend at the N-S axis. The plasma level of CPZ following its administration to psychiatric patients is 21.8 to 92.4 ng/mL [2]. It is known to interact with different biological macromolecules like DNA, RNA, and proteins [3,4]. The administration of some antipsychotic drugs produces adverse side-effects such as parkinsonism, cornea opacity, and respiratory troubles. The cause of many of the side-effects is still unclear, although it has been shown recently that some of these effects, e.g. cornea opacity, are not due to CPZ consumption [5]. CPZ is also known to induce hyperglycaemia and can inhibit insulin secretion in both normal patients and patients with latent diabetes mellitus, who are administered high acute doses of the drug [6]. The mechanism by which CPZ causes hyperglycaemia and inhibits insulin secretion is unknown.

Insulin is composed of 51 amino acids in two peptide chains (A and B) linked by two disulphide bonds. The three-dimensional structure of the insulin molecule, essentially the same in solution and in the solid phase, exists in two main conformations. These differ in the extent of the helix in the B-chain, which is governed by the presence of phenol or its derivatives. The intrinsic flexibility at the ends of the B-chain plays an important role in governing the physical and chemical stability of insulin. A variety of chemical changes affect the structure of insulin, resulting in denaturation, aggregation, and precipitation [7].

In the present study, we investigated a probable mechanism by which CPZ causes hyperglycaemia. Our results indicate that CPZ interacts with insulin causing aggregation and thus hyperglycaemia in patients administered the drug.

2. Materials and methods

CPZ was obtained as a gift from Sun Pharmaceuticals. Insulin from bovine pancreas, α-lactalbumin, and DTT were purchased from the Sigma Chemical Co. All other chemicals were of analytical grade.

An aqueous stock solution of CPZ was made up fresh before each set of experiments. Two absorption maxima were seen: one at 254 nm (ϵ = 33,200 $M^{-1}cm^{-1}$) and the other at 305 nm (ϵ = 4000 $M^{-1}cm^{-1}$). Solutions of CPZ up to a 1000 μM concentration obeyed Beer's law, and showed no significant absorbance near the visible wavelength region.

The kinetics of aggregation was followed by measuring the apparent absorption at 360 nm due to light scattering of 1-mL solutions of 0.4 mg insulin or 1.5 mg α-lactalbumin in the presence of 20 mM DTT or 200 μM CPZ in 10 mM

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Abbreviations: CPZ, Chlorpromazine; and DTT, Dithiothreitol.

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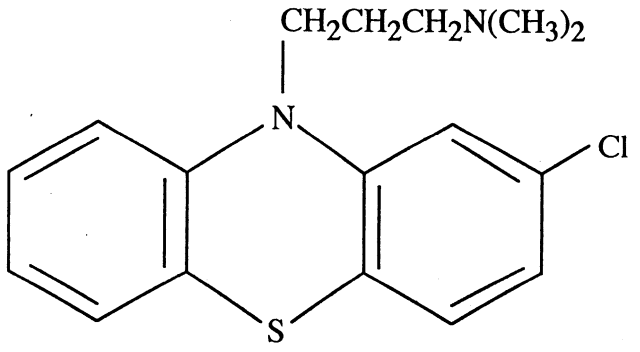

CHLORPROMAZINE

Fig. 1. Structure of chlorpromazine (CPZ).

phosphate buffer, pH 6.8. The measurements were performed at room temperature using a Shimadzu UV2401PC spectrophotometer equipped with a multicell transporter with a Peltier temperature regulator.

All fluorescence measurements were done in a Hitachi F-4200 spectrofluorimeter. Fluorescence emission spectra (290–500 nm) of insulin (0.4 mg/mL), CPZ (200 μM), and supernatants and pellets (dissolved in buffer with one drop of 4 M urea) obtained after centrifugation of the aggregated solution of 0.4 mg/mL of insulin and 200 μM CPZ interaction or 0.4 mg/mL of insulin and 20 mM DTT interaction, were obtained by exciting each sample at 275 nm. The excitation and emission bandpasses were kept at 5 nm. A 10 mM sodium phosphate buffer, pH 6.8, was used in all the fluorescence measurements. A blank scan was subtracted from all the sample scans.

HPLC size-exclusion studies were performed in a calibrated Synchropak GPC100 (total bed volume ~15 mL) column at a flow rate of 0.5 mL/min. Fifty microliters of sample (pellet or supernatant of either CPZ–insulin or DTT–insulin interaction) was injected into the column, and the signal was monitored by a diode array detector at 220 nm. Calibration of the column was done with bovine serum albumin (MW 66,000), α-lactalbumin (MW 14,300), mellitin (MW 2860), bradykinin (MW ~1000), and DTT (MW 154).

3. Results and discussion

Figure 2 shows the kinetics of insulin (0.4 mg/mL in 10 mM phosphate buffer, pH 6.8) aggregation by monitoring light scatter at 360 nm in the presence of 20 mM DTT (curve a) and 200 μM CPZ (curve b). Bovine insulin possesses two chains, A and B, linked together by disulphide bridges. The reduction of insulin by DTT leads to aggregation of the insulin B-chain [8], thus inactivating it. Aggregation was also noticed when 200 μM CPZ was added to the same amount of insulin. It is evident from the figure that the rate of aggregation of insulin by CPZ is much faster than that by DTT. In addition, the light scattering profile of the CPZ–insulin interaction (hyperbolic) was markedly different from that the of DTT–insulin interaction (sigmoidal).

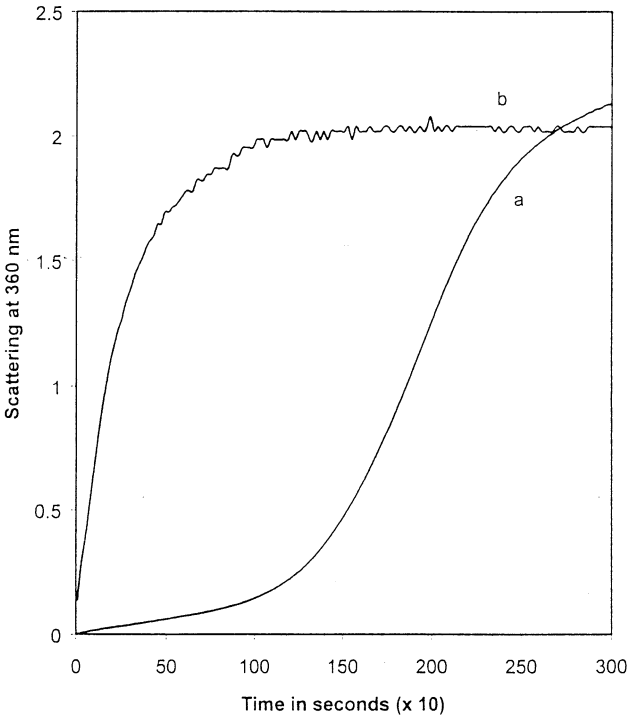

Fig. 2. Kinetic traces of insulin B-chain aggregation at 360 nm in the presence of (a) 20 mM DTT, and (b) 200 μM CPZ. The concentration of insulin in the assay system was 0.4 mg/mL.

Figure 3 shows the plot of the light scattering intensity at 360 nm caused by the interaction of 0.4 mg/mL of insulin and various concentrations of CPZ after 3000 sec. As shown, a much lower concentration of CPZ (50 μM) can also cause aggregation of insulin (Fig. 3). When insulin interacted with a lower concentration of CPZ, the light scattering intensity as monitored at 360 nm was less over time than when it interacted with a higher concentration of the drug. It is known that other antipsychotics also cause hyperglycaemia. We have also seen that other antipsychotics such as promethazine and trifluoperazine can induce light scatter in the presence of insulin (data not shown). In all instances, light scattering intensity varies depending upon the concentration of the individual drug. CPZ is one of the most widely used antipsychotics for the treatment of psychoses.

The tendency of insulin to undergo structural transformation, resulting in aggregation, has been one of the most intriguing and widely studied phenomena in relation to insulin stability. It has been known for many years that insulin A- and B-chains individually are inactive, but that the complete molecule can aggregate into dimers. Additionally, it is known that in the presence of sufficiently high

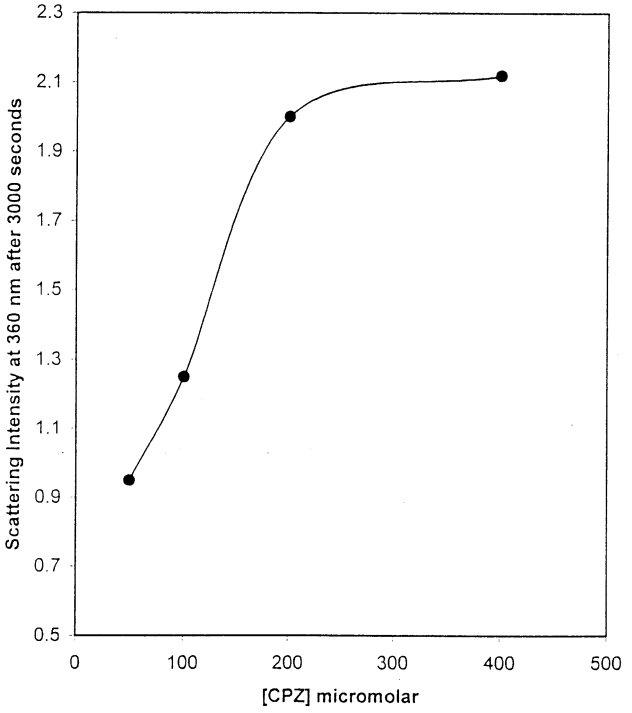

Fig. 3. Plot of the light scattering intensity caused by the interaction of 0.4 mg/mL of insulin with various concentrations of CPZ after 3000 sec.

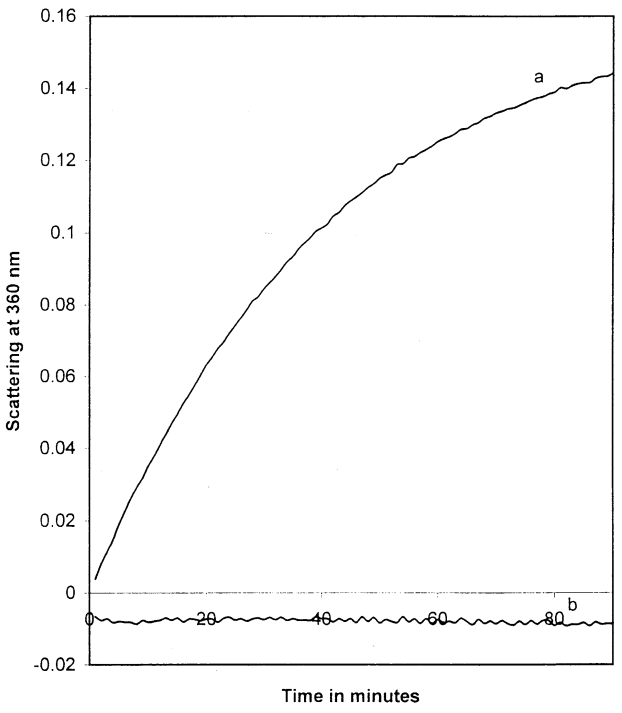

Fig. 4. Kinetic traces of bovine α-lactalbumin (1.5 mg/mL) at 360 nm in the presence of 20 mM DTT (a) and 200 μM CPZ (b).

concentrations of zinc, insulin can form hexamers. To rule out the possibility that the observed light scatter was caused by zinc-induced insulin aggregation, we repeated the experiments in the presence of 200 mM EDTA. We obtained results (data not shown) similar to those found in the absence of EDTA, thus negating the possibility that the observed light scatter in the presence of either DTT or CPZ may be due to metal ions contaminating the bovine insulin preparation. Another possibility of insulin aggregation is the formation of inactive insulin by reduction of the disulphide bonds whereby the insulin B-chain aggregates and the insulin A-chain remains in solution as occurs in the presence of DTT [8].

The obvious question we next tried to address is whether the phenomena of CPZ-induced and DTT-induced insulin aggregation are alike. To test whether CPZ can cause aggregation of other DTT-precipitating proteins with disulphide bridges, we measured the kinetics of α-lactalbumin light scattering in the presence of DTT and CPZ. α-Lactalbumin is an ~14 kDa protein possessing disulphide linkages. It is known that DTT causes aggregation of α-lactalbumin by reduction of disulphide bonds in a manner similar to insulin [9]. Figure 4 shows the light scattering profile of α-lactalbumin (1.5 mg/mL in 10 mM phosphate buffer, pH 6.8) in the presence of 20 mM DTT and 200 μM CPZ. From the figure, it is evident that although α-lactalbumin aggregates in the presence of 20 mM DTT, it does not aggregate at the plasma concentration of CPZ (200 μM). α-Lactalbumin did not precipitate even in the presence of 400 μM CPZ. CPZ caused aggregation of α-lactalbumin only at a very high concentration (1000 μM) (data not shown). Since we are trying to explain a possible mechanism for hyperglycaemia in patients, we have limited our studies to within the plasma concentrations of CPZ. With insulin, 200 μM CPZ caused instantaneous precipitation that could be detected by monitoring light scatter at 360 nm, but this concentration of CPZ was not sufficient to cause aggregation of 1.5 mg/mL of α-lactalbumin. Precipitation occurred only if the protein was unfolded as a result of the formation of insoluble aggregates. Therefore, at levels found in plasma, CPZ causes aggregation of insulin but not of α-lactalbumin.

To characterize the products of DTT–insulin and CPZ–insulin (supernatant and pellet) interactions, we scanned the fluorescence spectra of the products from 290–500 nm upon excitation at 275 nm (Fig. 5). Excitation and emission slits were kept at 5 nm each. The emission spectra of 200 μM CPZ and 0.4 mg/mL of insulin were also scanned as a control. Two hundred micromolar CPZ alone excited at 275 nm gave an emission maxima at 460 nm (curve c). Insulin alone excited at 275 nm (characteristic for tyrosine) had an emission maxima at 310 nm without any peak at 460 nm (curve i). The supernatant (curve 1) of the CPZ–insulin interaction showed two emission peaks: one at ~310 nm (characteristic for tyrosine residues) and the other at ~460 nm (characteristic of CPZ in solution). The pellet (curve 2) also showed two peaks: one at ~310 nm and the other at ~460 nm. In the case of DTT–insulin interaction, the supernatant (curve 3) and the pellet (curve 4) showed only one peak at ~310 nm, a characteristic of the tyrosine emission. Since DTT is not a fluorophore, no fluorescence signal is

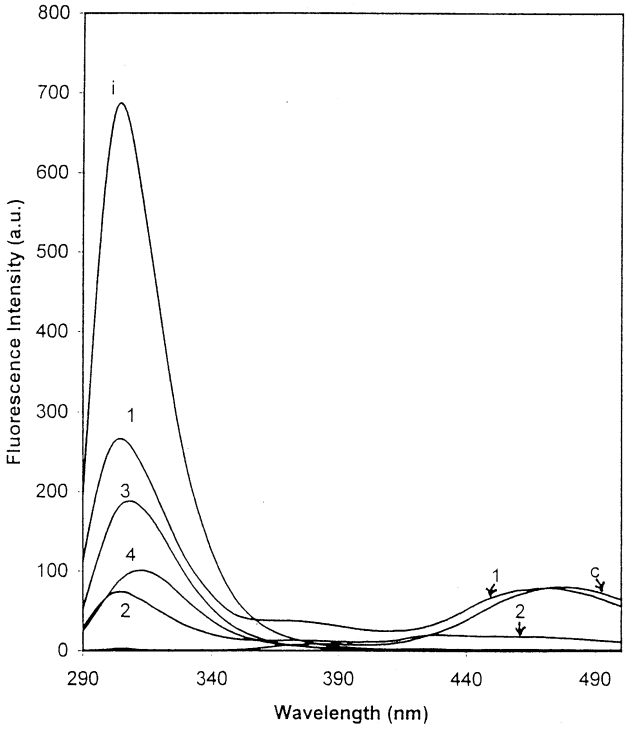

Fig. 5. Emission spectra (290–500 nm) of: 0.4 mg/mL of insulin (i); 200 μM CPZ (c); supernatant after centrifugation of 200 μM CPZ with 0.4 mg/mL of insulin [1] and pellet after centrifugation of 200 μM CPZ with 0.4 mg/mL of insulin [2]; supernatant after centrifugation of 20 mM DTT with 0.4 mg/mL of insulin [3] and pellet after centrifugation of 20 mM DTT with 0.4 mg/mL of insulin [4]. Excitation and emission bandpasses were kept at 5 nm each. The samples were excited at 275 nm.

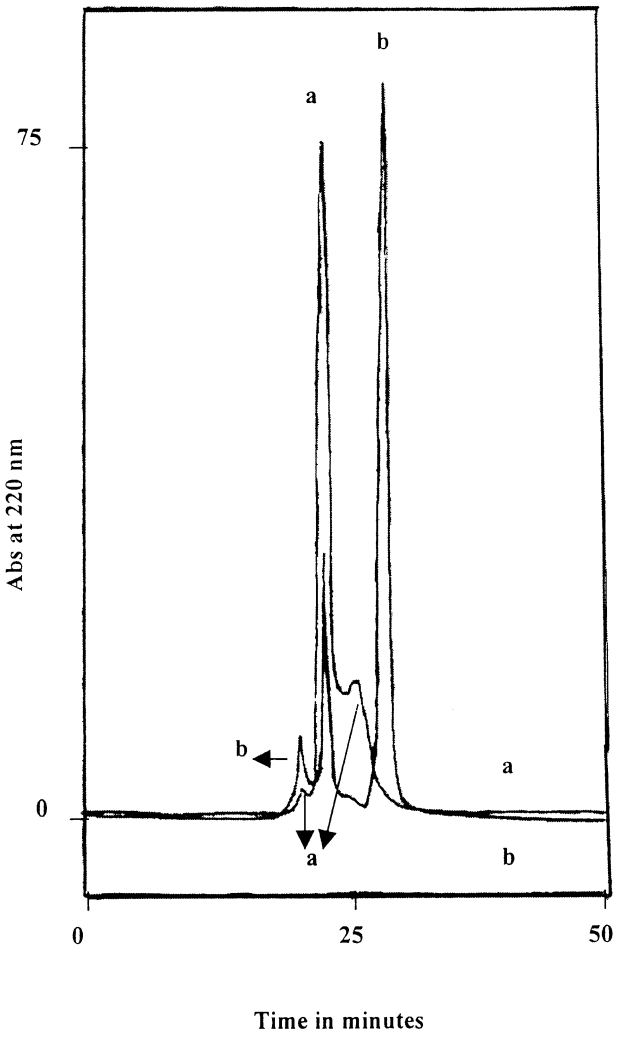

Fig. 6. HPLC gel-filtration profile for the supernatants of CPZ–insulin (a) and DTT–insulin (b) interactions.

obtained for DTT. It is known that reduction of insulin with DTT leads to aggregation of the B-chain [8]. Thus, in the case of the DTT–insulin interaction, the pellet contains only the B-chain of insulin and, therefore, the supernatant contains non-aggregated insulin A-chain and unreacted insulin. The case for the CPZ–insulin reaction may be similar, excluding the contribution from the drug. Therefore, apart from the CPZ signal at 460 nm in the case of CPZ–insulin interaction, it seems that the fluorescence spectra of the products (supernatant and pellet) of DTT–insulin and CPZ–insulin interactions are similar.

We also analyzed the approximate mass of the products of both DTT–insulin and CPZ–insulin interactions by size-exclusion HPLC. Insulin (0.4 mg/mL) was reacted separately with 20 mM DTT and with 200 μM CPZ for 24 hr at room temperature. The pellets of both of the interactions were dissolved in 10 mM phosphate buffer, pH 6.8, containing 4 M urea. Fifty microliters of each pellet and supernatant was injected into the HPLC column, and the elution profile was detected by means of a diode array detector at 220 nm. The calibration of the column (data not shown) was done with bovine serum albumin (MW 66,000), α-lactalbumin (MW 14,300), mellitin (MW 2860), bradykinin (MW ~1000), and DTT (MW 154). The supernatant of the insulin–DTT reaction (Fig. 6) showed peaks at ~20.3 min (corresponding to total insulin –total insulin shows a peak at this position), ~23.5 min (corresponding to insulin A-chain –insulin A-chain separately showed a peak at this position corresponding to a molecular mass of ~3000), and ~29.0 min (corresponding approximately to the molecular weight of DTT). The supernatant of the insulin–CPZ reaction showed a very small peak at ~20 min (corresponding to insulin) and two major peaks at 23.8 min (corresponding probably to the insulin A-chain) and 26.4 min (corresponding to CPZ). So, the supernatants of both CPZ–insulin and DTT–insulin interactions (Fig. 6) showed common peaks corresponding to total insulin (insulin that had not been degraded) and insulin A-chain, apart from the CPZ peak or the DTT peak of CPZ–insulin and DTT–insulin interactions, respectively. The pellet of the CPZ–insulin reaction (Fig. 7) showed a peak at ~25.8 min, whereas the pellet of the insulin–DTT reaction gave peaks at ~25.4 and ~28.6 min (corresponding to DTT). The presence of urea may have caused the degradation of the insulin B-chains, which

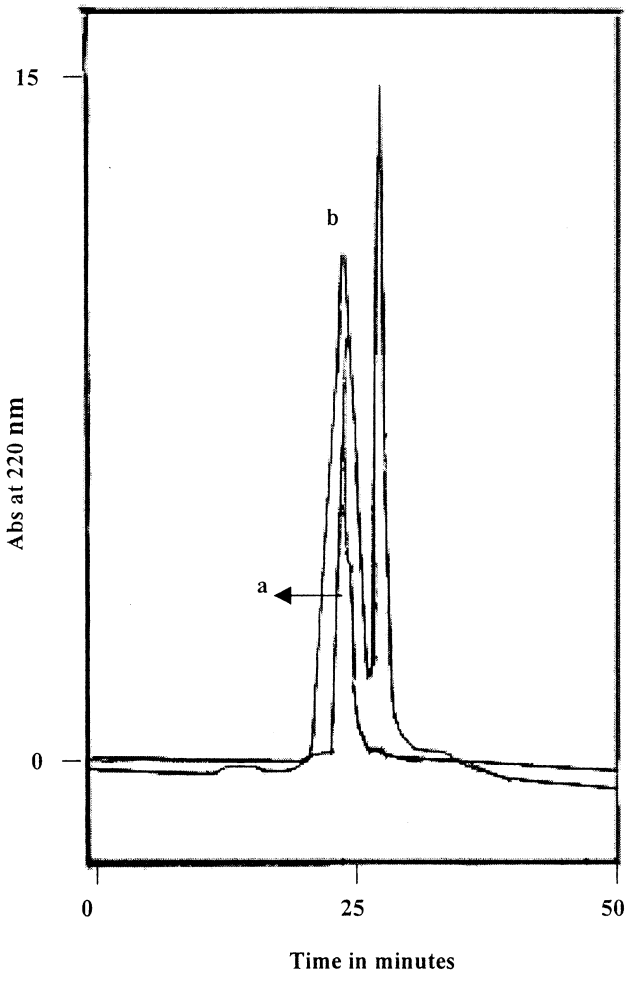

Fig. 7. HPLC gel-filtration profile for the pellets of CPZ–insulin (a) and DTT–insulin (b) interactions.

gave a peak at around 25 min (corresponding to ~1584 Da). In the case of the pellet from the CPZ–insulin interaction, the contribution from CPZ merged in the same region (25.8 min). In addition to the peak at 25.4 min, the pellet of the DTT–insulin interaction also showed a peak at around 29 min (representing DTT). Therefore, the pellets of both the CPZ–insulin and DTT–insulin interactions show a common peak at ~26 min leaving the contributions from either CPZ or DTT. Taken collectively, it appears that the products (supernatant and pellet) of both DTT–insulin and CPZ–insulin interactions are similar, excluding the contributions of DTT or CPZ, respectively.

Our experiments therefore reveal that CPZ possibly causes aggregation of insulin through the reduction of disulphide bonds (like DTT). This, in turn, causes insulin to become inactive and hence induces hyperglycaemia. We also speculate that during the reaction process CPZ itself is oxidized, probably via its sulphur atom. The exact mechanism awaits further study. We therefore presume that high doses of CPZ can interact with insulin (by causing aggregation of insulin), thereby leading to loss of its function and subsequent hyperglycaemia.

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