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EVALUATION OF THE EFFICACY OF D-PENICILLAMINE AND TRIENTINE AS COPPER CHELATORS USING AN *IN VITRO* TECHNIQUE INVOLVING OVINE RED BLOOD CELLS

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ABSTRACT

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An *in vitro* technique for haemolysing ovine red blood cells with copper sulphate was standardized to induce *c* 50 % haemolysis with 0,5 mM $CuSO_4$ after incubation for 14 h at 38 °C. This technique was then applied to test the efficacy of trientine and d-penicillamine in preventing haemolysis. Trientine concentrations of 0,5; 1,0 and 1,5 mM were found to be the most effective ($P<0,05$) in reducing copper-induced haemolysis. One and 1,5 mM concentrations of d-penicillamine were also effective ($P<0,05$), but in this experiment a 0,5 mM concentration failed to protect the erythrocytes.

INTRODUCTION

In South Africa chronic copper poisoning of sheep, known as enzootic icterus or "geelsiekte", occurs in large parts of the Karoo and southern Orange Free State (Bath, 1979; Kellerman, Coetzer & Naudé, 1988). Chronic ovine copper poisoning has 2 distinct phases *viz.* accumulation of copper in the tissues (prehaemolytic phase) and a haemolytic crisis (haemolytic phase). Copper accumulates in a wide variety of tissues, particularly in the liver. The period of accumulation of copper in tissues varies from a few weeks to months, during which time the animal is clinically normal (Howell & Gooneratne, 1987; Todd, 1962). However, as the liver can only store a finite amount of the element, excess copper is released into the circulation to cause a haemolytic crisis. The plasma copper concentration increases markedly 1 to 2 days before onset of a haemolytic crisis (Todd, 1962). Stress has been suggested as a factor that contributes to the sudden release of copper from the liver into the circulation (Arora, Anderson, Bucht, Frank & Kronevi, 1977). During the haemolytic phase, sheep become listless, lose their appetite, and have elevated respiratory and heart rates. Extensive haemolysis results in haemoglobinuria and icterus. The scleral blood vessels are discoloured chocolate-brown indicating methaemoglobinaemia (Bath, 1979; Howell & Gooneratne, 1987; Todd, 1962). A haemolytic crisis may be fatal within hours, with most animals dying within 1 to 4 days (Bath, 1979; Todd, 1962).

The *in vitro* copper haemolysis technique is a very effective model for simulating a copper-induced haemolytic crisis in various domestic animal species and man (Aaseth, Skaug & Alexander, 1984; Asano & Hokari, 1986; Sivertsen, 1980). In these studies, fresh red blood cells (rbc) were washed and incubated for up to 24 h in buffered saline solutions containing different concentrations of copper. Haemolysis was quantified by measuring the concentration of free haemoglobin in the supernatant with the aid of Drabkin's cyanmethaemoglobin method (Aaseth *et al.*, 1984; Asano & Hokari, 1986).

Human rbc incubated for 4 h with 0,3 mM $CuSO_4$ underwent approximately 15 % haemolysis. The haemolysis was slow during the first 2 h, but accelarated thereafter due to membrane damage. Spontaneous haemolysis in the absence of copper was 3 % (range 2–4 %) (Aaseth *et al.*, 1984). These authors also reported that d-penicillamine and triethylene tetramine (trientine) reduced *in vitro* copper-induced haemolysis.

Asano & Hokari (1986) incubated bovine rbc for varying periods in 0,1 or 0,5 mM $CuSO_4$. The percentage haemolysis increased relatively slowly during the first 12 h of incubation, but progressed more rapidly in the second 12 h. After 24 h incubation the haemolysis reached 30 and 80 % with 0,1 and 0,5 mM $CuSO_4$, respectively.

D-penicillamine and trientine are used as copper chelators in human medicine (Dubois, Rodgerson & Hambidge, 1990). Trientine was shown to be an effective alternative copper chelating agent in the treatment of Wilson's disease (hepatolenticular degeneration) in humans (Dubois *et al.*, 1990).

According to Soli, Froslie & Aaseth (1978), d-penicillamine has a copper mobilising effect and increased the urinary copper excretion in sheep. 2,3,2-Tetramine tetrahydrochloride (a product similar to trientine) was also found to be a safe and effective chelating agent in the treatment of copper hepatotoxicosis in Bedlington Terriers (Twedt, Hunsaker & Allen, 1988).

The objectives of this study were: (a) to standardize an *in vitro* technique so that moderate haemolysis of sheep rbc is induced at various copper sulphate concentrations in a reasonable incubation period, and (b) to evaluate the efficacy of the chelating agents, d-penicillamine and trientine, in reducing copper-induced haemolysis of sheep rbc.

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MATERIALS AND METHODS

Animals

Blood was drawn from clinically normal adult South African Mutton Merino ewes (n=3) on a standard vaccination and deworming programme. The animals were kept in paddocks and fed dry lucerne hay with water *ad libitum*. The copper, iron and zinc content of the lucerne was determined by means of a standard atomic absorption spectrophotometric technique.

Collection of red blood cells

Blood was periodically drawn from the ewes in 5 mℓ evacuated collection tubes containing citrate anticoagulant.

Washing of red blood cells

Within half an hour of collection, the blood was centrifuged (2 000 rpm for 10 min at 7 °C) and the plasma, white blood cells and platelets discarded. The packed rbc were then washed 3 times with 0,9 % saline containing 1 mM glucose and 5 mM tris, buffered to pH 7,4 with HCl (Aaseth *et al.*, 1984).

Determination of optimal incubation period

The packed rbc were pooled and mixed thoroughly. To determine spontaneous haemolysis, 0,5 mℓ packed rbc were resuspended in 4,5 mℓ saline/tris buffer without glucose and incubated. Glucose was omitted from the tris buffered solution as it is known to significantly depress copper-induced haemolysis (Asano & Hokari, 1986; C. J. Botha, unpublished data, 1990). To induce total haemolysis, 1 drop to Triton X-100[1] was added to the rbc suspension (Aaseth *et al.*, 1984). Haemolysis induced by 0,5 mM $CuSO_4$ was determined by adding 400 µg copper sulphate[2] to 4,5 mℓ of the saline/tris buffer, before the addition of 0,5 mℓ of packed rbc. The test tubes with rbc suspensions (mean haemoglobin concentration 22,9 [19,0–27,0] g/ℓ) were incubated in a horizontal position for 12, 16 or 20 h at 38 °C in a reciprocal shaking (60 strokes/min) waterbath. In each case, a tube containing a rbc suspension with copper sulphate was incubated together with one without copper sulphate (spontaneous haemolysis) and one to which Triton X-100 had been added (total haemolysis). The experiment was repeated 3 times.

Determination of optimal copper sulphate concentrations

Following the establishment of an appropriate incubation period, 0,5 mℓ of packed rbc were incubated in 4,5 mℓ of saline/tris buffer containing 0,4; 0,5; 0,7 and 1,0 mM $CuSO_4$. The design of the trial was similar to that for determining the optimal incubation period.

Quantification of haemolysis

The copper-induced haemolysis was quantified by measuring the haemoglobin concentration in 0,2 mℓ of the supernatant, using the Drabkin's cyanmethaemoglobin method (Drabkin & Austin, 1935) and a Pye Unicam SP 1800 Ultraviolet spectrophotometer. The percentage haemolysis of each sample was calculated from the haemoglobin concentration measured, relative to that of copper sulphate-free rbc suspensions to which Triton X-100 had been added (total haemolysis) (Aaseth *et al.*, 1984; Asano & Hokari, 1986). Spontaneous haemolysis was determined after incubation and subtracted from that measured for each sample to calculate the percentage of copper-induced haemolysis.

Incubation of red blood cells to evaluate efficacy of chelators

The efficacy of d-penicillamine hydrochloride[3] and triethylene tetramine tetrahydrochloride (trientine)[3] in preventing copper-induced haemolysis was evaluated. Samples of 3 different concentrations of each chelator (0,5; 1,0 and 1,5 mM) were added to the rbc suspensions at various concentrations of copper sulphate (0,40; 0,45; 0,50; 0,60 mM). In addition, trientine was evaluated at copper concentrations of 0,8; 1,0 and 1,2 mM. The rbc/copper sulphate/chelator-suspensions were incubated together with rbc/copper sulphate-suspensions (controls) and suspensions measuring spontaneous and total haemolysis *(vide supra)*. Only one chelator at 3 different concentrations was assayed at a time. The mean percentage haemolysis for every repetition was calculated and the mean for the whole experiment determined. The treatments together with their controls (16 pairs) were incubated with tubes measuring spontaneous haemolysis (1 pair) and total haemolysis (1 pair). Five trials in all were conducted with penicillamine and 4 with trientine.

Five trials to test the haemolytic effect of the 2 chelators at the concentrations used in this experiment were also carried out.

Statistical analysis of data

The data was subjected to an analysis of variance for a factorial design (copper concentration and chelator concentration). The significance of the main effects and interaction between main effects were tested at a confidence level of 95 % ($P<0,05$). All analyses were performed using PC-SAS[4].

The differences between main effect levels were tested with the aid of Scheffe's means separation test. The significance of the differences between the degree of haemolysis caused by either of the chelators were compared with that of spontaneous haemolysis (control) by way of Dunnett's means separation test. Percentage data were transformed using an Arcsin transformation prior to statistical analyses. Means and standard deviations were calculated according to standard statistical techniques.

RESULTS

Feed analysis

The results of the mineral analyses performed on the feed were Cu 2,5 ppm, Fe 94 ppm and Zn 14 ppm (dry matter basis).

Standardization of in vitro *technique*

Spontaneous haemolysis (n=3) after 12 h ranged from 4,88 to 13,11 % (mean 8,17; SD 4,36); after

[1] Sigma
[2] Anhydrous, Merck, AR
[3] Sigma
[4] SAS Institute Incorporated, Cary, North Carolina, USA

16 h from 7,58 to 15,04 % (mean 11,01; SD 3,77) and after 20 h from 9,54 to 18,98 % (mean 15,02; SD 4,9).

Haemolysis induced by 0,5 mM $CuSO_4$ (n=3) at 12; 16 and 20 h is demonstrated in Fig. 1. After 12; 16 and 20 h the mean percentage copper-induced haemolysis were 34,28 % (SD 11,77); 63,43 % (SD 5,05) and 74,36 % (SD 11,33), respectively. After 14 h incubation the interpolated copper-induced haemolysis was *c* 50 % (Fig. 1). Fourteen hours was thus selected as a reasonable incubation period because 50 % haemolysis was taken as a good indicator of copper sulphate-induced haemolysis.

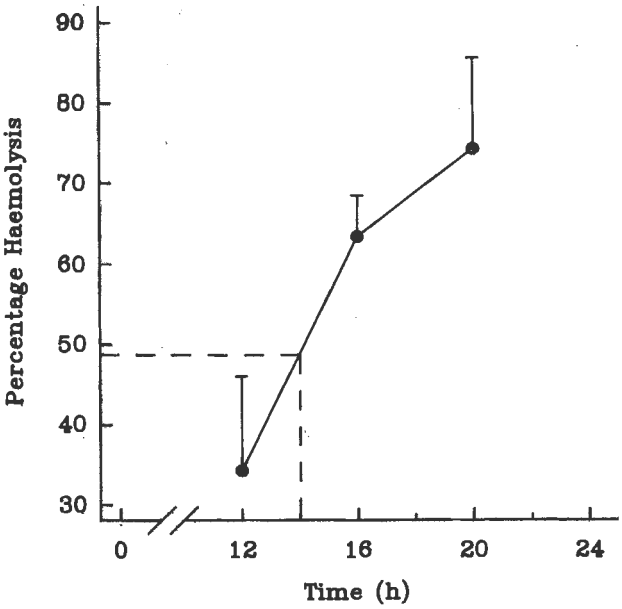

FIG. 1 Copper-induced haemolysis at 12, 16 and 20 h without glucose

After 14 h incubation the estimated mean spontaneous haemolysis from 5 replicates each consisting of 4–6 observations (n=27) ranged from 8,24 to 15,15 % (mean 12,3; SD 2,56). The mean haemolysis induced by 0,5 mM $CuSO_4$ estimated from 5 similar replicates (n=27) was 51,67 % (SD 6,14); the mean haemolysis induced by 0,4 mM $CuSO_4$ estimated from 3 replicates of 6 samples each (n=18) was 23,75 % (SD 11,05). Four replicates, each consisting of 4–6 observations (n=22), to determine haemolysis induced with 0,7 mM $CuSO_4$ resulted in mean haemolysis of 66,29 % (SD 5,11). The mean haemolysis induced by 1,0 mM $CuSO_4$ estimated from 4 replicates, each consisting of 5–6 samples (n=23), was 64,83 % (SD 6,83).

Dose-response effect

The effects of d-penicillamine and trientine on haemolysis of rbc incubated for 14 h with varying concentrations of copper sulphate are depicted in Fig. 2 and 3, respectively.

One mM and 1,5 mM d-penicillamine concentrations significantly (P<0,05) reduced the copper-induced haemolysis of rbc compared to the control. The 0,5 mM d-penicillamine concentration contrarily caused significantly more haemolysis than the control. However, the antihaemolytic effects of 1,0 and 1,5 mM d-penicillamine on copper induced haemolysis did not significantly differ from one another (Fig. 2).

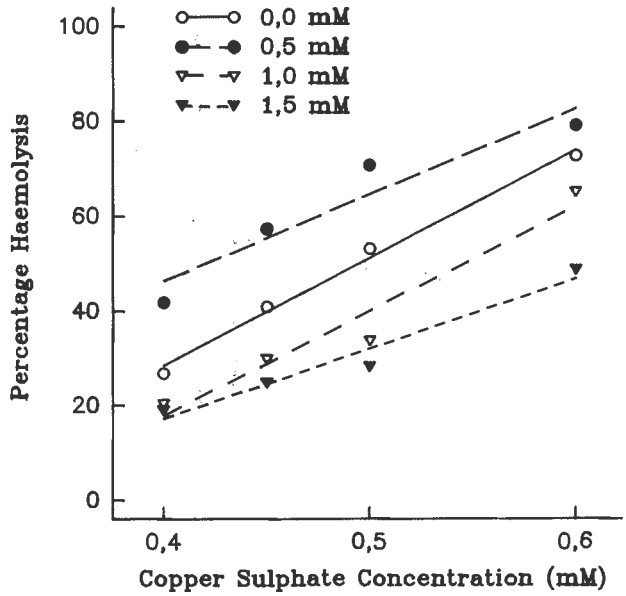

FIG. 2 The efficacy of d-penicillamine in reducing copper-induced haemolysis

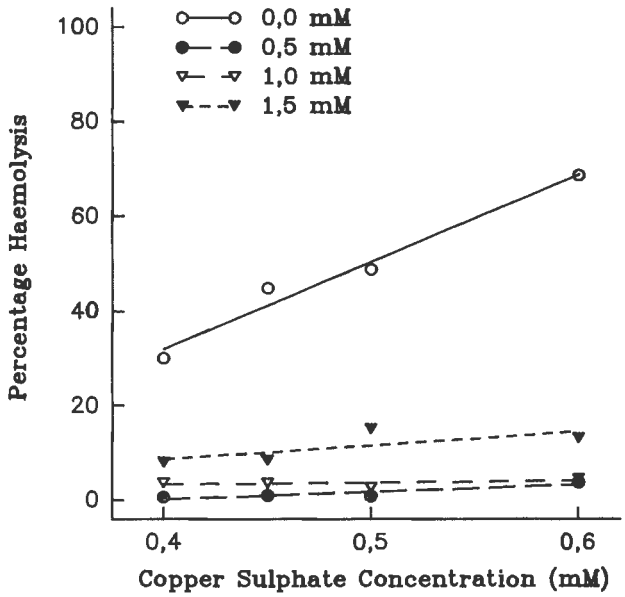

FIG. 3 The efficacy of trientine in reducing copper-induced haemolysis

All the trientine solutions (0,5; 1,0 and 1,5 mM) significantly (P<0,05) reduced the copper-induced haemolysis compared with the control. Here again, no statistically significant difference could be demonstrated between copper-induced haemolysis in the presence of 1,0 or 1,5 mM trientine (Fig. 3). However, higher copper sulphate concentrations than those used with d-penicillamine were required to cause a similar degree of haemolysis (Fig. 5).

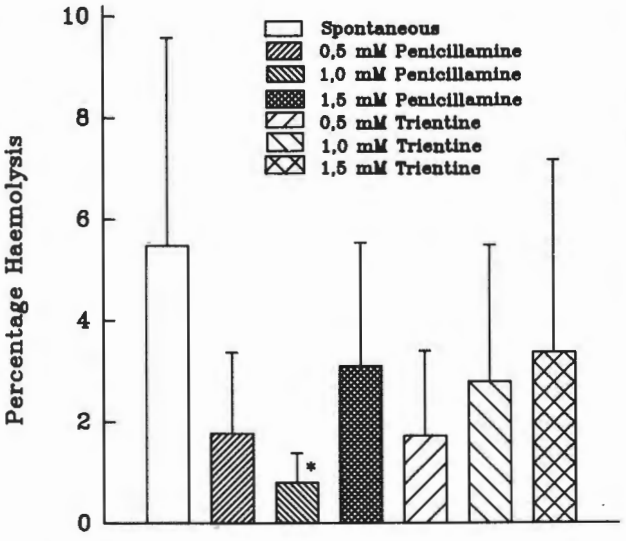

FIG. 4 Haemolysis induced by 3 different chelator concentrations compared to the spontaneous haemolysis

Both chelators at 0,5–1,5 mM caused only a slight haemolysis on incubation with rbc (chelator-induced haemolysis). When comparing the 2 chelators, there were no statistical significant differences in haemolysis. The mean d-penicillamine-induced haemolysis (n=5) ranged from 0,8–3,1 % and that of trientine (n=5) from 1,72 to 3,37 % at these concentrations (Fig. 4). After 14 h incubation the spontaneous haemolysis (n=5) varied from 2,13 to 11,8 % (mean 5,48; SD 4,1). D-penicillamine-induced haemolysis was significantly lower than spontaneous haemolysis. When the haemolysis induced by the different concentrations of d-penicillamine were compared with spontaneous haemolysis, the 1,0 mM concentration caused the least haemolysis ($P<0,05$) (Fig. 4).

The effects of 1,0 and 1,5 mM concentrations of both chelators are depicted in Fig. 5. The trientine curves are shifted further to the right than are those of d-penicillamine. Furthermore, 0,5 mM trientine provided better protection against haemolysis than the highest penicillamine concentration.

Discussion

The suitability of a 14 h incubation period as determined during the standardization study was confirmed by the dose-response studies. After 14 h of incubation with 0,5 mM $CuSO_4$ the mean haemolysis recorded in 9 trials was 51,24 %. This closely corresponds with the expected haemolysis at 14 h interpolated from Fig. 1 and the actual haemolysis of 51,67 % recorded during the trials to determine the optimal copper sulphate concentrations.

In this particular investigation, trientine, on an equimolar basis, was found to be more potent than

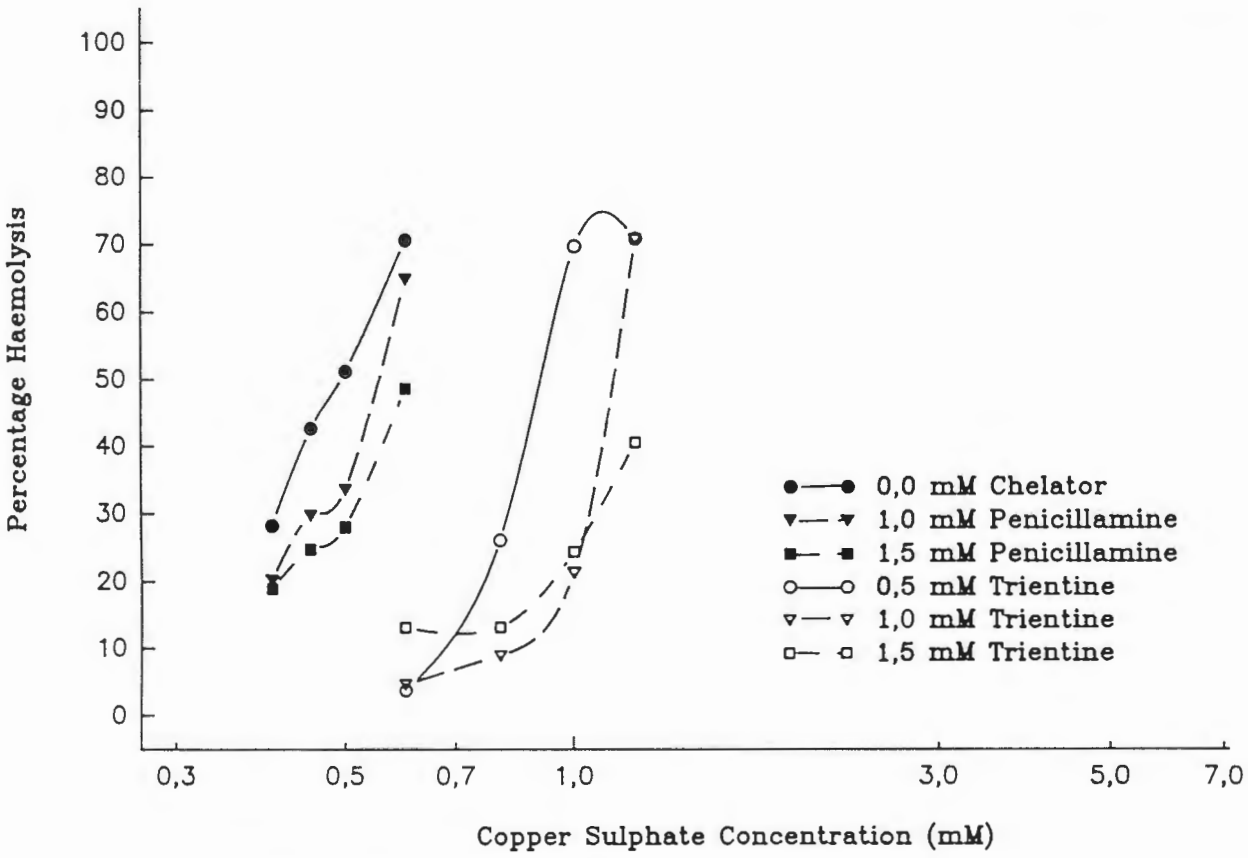

FIG. 5 Dose-response curves depicting chemical antagonism

d-penicillamine in preventing copper sulphate-induced haemolysis. Potency is usually defined by the position of the curve on the log dose-response plot; if the curve is closer to the ordinate if denotes greater potency. Althouth the d-penicillamine curve lies closer to the ordinate it is not more potent, because the abscissa represents the logarithm of the copper sulphate concentration. In other words in the presence of trientine, a higher copper sulphate concentration was necessary to cause a moderate amount of haemolysis (Fig. 5).

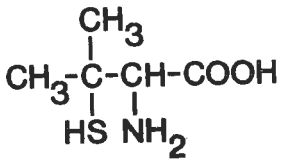

FIG. 6 D-penicillamine

A possible explanation for the greater potency of trientine may lie in its chemical structure. Pearson (1968) as cited by Aaseth *et al.* (1984) states that copper ions have an extremely high affinity for SH-groups and a very high affinity for NH_2-groups. According to Correia & Becker (1987) a single molecule of copper may be chelated by 2 molecules of d-penicillamine (Fig. 6).

The trientine molecule is a small polymer containing 2 primary and 2 secondary amine groups. It is possible that the greater number of binding sites per molecule, in conjunction with the very high affinity of copper for the NH_2-groups, could account for its greater potency (Fig. 7).

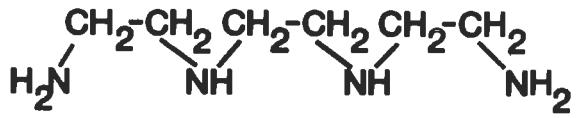

FIG. 7 Trientine

The d-penicillamine and trientine curves lie parallel to each other and hence it can be deducted that the mechanism of action of the 2 chelators is most probably similar (Fig. 5). Both are believed to act as chemical antagonists and not as pharmacological antagonists (affinity for tissue receptors). The chemical antagonists (d-penicillamine and triéntine) shift the agonist (copper sulphate) curve parallel to the right.

The findings of Aaseth *et al.* (1984) using human rbc, that a 15 % haemolysis with 0,3 mM $CuSO_4$ occurred after 4 h incubation, corresponded with those of the current investigation. Using sheep rbc and a similar *in vitro* method in the standardization (n=3) and dose-response (n=9) studies, 23,75 % and 28,22 % haemolysis were respectively induced with 0,4 mM $CuSO_4$ after 14 h. Spontaneous haemolysis in the entire experiment ranged between 0,97–15,15 % as compared with 2–4 % reported by Aaseth *et al.* (1984). The variation in the degree of spontaneous haemolysis in this experiment can probably be explained by the nature of the rbc, as sheep erythrocytes are noted for their fragility (Ruckebusch, Phaneuf & Dunlop, 1991). It was concluded that both d-penicillamine and trientine reduced copper-induced haemolysis of sheep rbc, a finding consistent with that of Aaseth *et al.* (1984) with human rbc.

The reason why 0,5 mM d-penicillamine caused significantly more haemolysis compared with the control is unknown.

Trientine has been shown in this study to be an effective *in vitro* chelator of copper and its efficacy in preventing haemolysis during chronic ovine copper poisoning will be investigated.

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