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EFFECTS OF DIFFERENT ANTICOAGULANTS ON DETERMINATION OF ERYTHROCYTE GLUTATHIONE PEROXIDASE

By

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HUSSEIN, KAMAL S. M. and BERNT-E. V. JONES: *Effect of different anticoagulants on determination of erythrocyte glutathione peroxidase.* Acta vet. scand. 1981, *22*, 472—479. — Glutathione peroxidase (GSH-Px) was determined in whole blood from cows, goats and horses using cumenehydroperoxide as substrate. Heparin was found to be the most suitable anticoagulant. The highest activities of GSH-Px were found with high concentrations of heparin in the blood samples (1000 and 1250 IU/ml of blood). Sodium fluoride and especially EDTA and sodium citrate gave lower activities of the enzyme.

Storage of the blood samples at room temperature (~20°C) or in a refrigerator (~5°C) for 3 days resulted in significantly lower activities of the enzyme, especially in horse blood.

glutathione peroxidase; anticoagulants; storage; cattle; horse; goat.

Glutathione peroxidase (glutathione hydrogen peroxide oxidoreductase, GSH-Px, E.C.1.11.1.9.) is a selenioenzyme, so far the only one identified in mammalian species (*Anon.* 1981). Isolation of crystalline GSH-Px from bovine and ovine erythrocytes has shown 4 moles of selenium on 1 mole of GSH-Px (*Hafeman et al.* 1974, *Oh et al.* 1974, *Smith et al.* 1974). Selenium is present as selenocystein in the enzyme (*Rotruck et al.* 1973). Good correlation between the selenium concentration in blood and the activity of GSH-Px in erythrocytes has been found for cattle, sheep, pigs, horses and humans, thus enabling this enzyme to be used as an indicator of the selenium status in animals (*Wilson et al.* 1976, *Jørgensen et al.* 1977, *Thompson et al.* 1977, *Anderson et al.* 1978, *Caple et al.* 1978). Furthermore, *Perona et al.* (1978) found that addition of selenium stimulates the activity of GSH-Px in human erythrocytes both in vivo and in vitro.

The GSH-Px of the erythrocytes acts as an integral part of their anti-oxidative system (*Mills & Randall* 1958). Especially the breakdown of hemoglobin is protected by this system. There is, however, also glutathione peroxidase activity that is not selenium dependant. Also this enzyme is of importance for the protection of tissues in vivo from oxidative changes (*Lawrence & Burk* 1976, 1978).

The present study was undertaken to check the effect of different anticoagulants on the determination of GSH-Px in whole blood from cattle, goats and horses. The effect on the preserved blood of storage at different temperatures was also tested.

MATERIAL AND METHODS

GSH-Px was determined according to a method described by *Paglia & Valentine* (1967) using cumenehydroperoxide as substrate. The activity determinations were performed at 37°C on a LKB 8600 Reaction Rate Analyzer (LKB, Bromma, Sweden).

Experiment 1

Blood samples were obtained from 5 adult cows (Swedish Red and White Breed) and 5 adult horses (standardbred trotters). The blood was collected with a syringe and put into different tubes to give 2 final concentrations of heparin, 15 IU and 500 IU per ml of blood, and also into tubes with EDTA (70 mmol/ml of blood) and sodium fluoride (0.1 mmol/ml of blood). Blood was also obtained from 14 goats (Swedish Land Breed). The blood from the goats was anticoagulated with heparin (15 IU/ml), EDTA (70 mmol/ml), sodium fluoride (0.1 mmol/ml) and sodium citrate (10 mmol/ml). The samples were analysed after immediate freezing of the hemolysate and after 3 days of storage at room temperature (~ 20°C) of the blood samples. The samples from the goats were also analysed after 3 days of storage in a refrigerator (~ 5°C). All samples were analysed at the same time to avoid between-run differences.

Duplicate determinations of the samples from cows and horses were used to calculate the standard error of a single determination using the formula:

$$s = \sqrt{\frac{\Sigma d^2}{2n}}$$

where d is the difference between duplicate determinations and n is their number.

A reserve of blood from a cow was kept in aliquots at —20°C for 6 months and used to check the within and between-assay variations. The mean and standard deviations of these samples were calculated and used to determine the coefficient of variation.

A paired t-test was used to compare the results.

Experiment 2

Blood from 6 female goats was used to check the effect of different amounts of heparin. The blood was collected with a final concentration of 125, 250, 500, 1000 and 1250 IU of heparin per ml of blood. These samples were analysed after immediate freezing and after storage for 4 days at room temperature (~ 20°C).

A paired t-test was used to compare the results. The differences were compared for $P > 0.95$ and $P > 0.99$.

RESULTS

Experiment 1

The blood preserved with heparin showed higher activities of GSH-Px than that preserved with EDTA or fluoride in both cows and horses. There was no significant difference between the two concentration of heparin used in cows and horses, but the 500 IU/ml samples showed higher values. Also in goats the heparin-preserved blood gave the highest readings of GSH-Px. The means and ranges of the results are given in Table 1.

Storage of the blood samples at room temperature for 3 days gave decreased activities with all anticoagulants (Table 1) in cows, horses and goats. The activity of GSH-Px decreased especially in horses.

Only the heparin-preserved blood from 4 goats was stored for 6 days at both room temperature and in the refrigerator. There was a significant decrease in GSH-Px activity in all these samples compared to the immediately-frozen heparin hemolysate.

The determination of GSH-Px in 23 duplicate samples with the range 162—489 μkat/l (mean 326.4 μkat/l) gave a coefficient of variation of 2.4 %.

In the range 500—1477 μkat/l (mean 808.3 μkat/l) the coeffi-

Table 1. Effects of different anticoagulants and storage on the determination of GSH-Px in erythrocytes from cows, horses and goats.

Animals	Immediate freezing of the hemolysate				3 days of storage at ~ 20°C			
	Heparin 15 IU/ml	Heparin 500 IU/ml	EDTA 70 mmol/ml	NaF 0.1 mmol/ml	Heparin 15 IU/ml	Heparin 500 IU/ml	EDTA 70 mmol/ml	NaF 0.1 mmol/ml
Cows (n=5)								
mean μkat/l	503 a	576 b, c	428 a, b, d—g	504	508 c, d	528 e	534 f	529 g
range	234—802	240—971	162—713	213—741	199—867	225—928	232—931	207—852
Horses (n=5)								
mean μkat/l	785	819, h, A—D	738 h	778 i, j, E	717 i, A	718 B, E	724 j, C	723 D
range	338—1428	391—1477	384—1407	383—1391	342—1285	339—1309	335—1340	350—1319
Goats (n=14)								
mean μkat/l	389 k, l, F, G	—	373 m, F	373 G	435 (n=4)	—	371 k (n=4)	—
range	218—603		218—589	210—584	313—622		213—572	
					3 days of storage at ~ 5°C			
Goats								
mean μkat/l					422 l (n=4)	—	422 (n=3)	375 m (n=4)
range					308—580		233—633	256—489

Note that the stored samples from the goats are a subsample of the immediately frozen samples and the means therefore are not directly comparable.
Figures followed by the same letter are significantly different in paired t-test comparisons. Upper case letters denote $P > 0.99$ and lower case letters $P > 0.95$.

Table 2. The effect of different concentrations of heparin on the determination of GSH-Px in goats blood (n=6) immediately frozen for analysis and stored at room temperature (~20°C) for 4 days.

	Heparin concentration, IU/ml of blood				
	125	250	500	1000	1250
Immediately frozen for analysis					
mean μkat/l	435 a, A—E	435 b, F—J	435 c, K—O	454 a, b, c, P—T	441 d, e, U—X
range	335—625	350—609	356—661	363—660	347—653
Stored at ~ 20°C for 4 days					
mean μkat/l	388 d, A, F, K, P	395 e, B, G, L, Q	379 f, C, H, R, U, Y	395 D, I, N, S, V, Y	393 f, E, J, O, T, X
range	292—528	325—531	292—547	305—576	308—547

Figures followed by the same letter are significantly different in paired t-test comparisons. Upper case letters denote $P > 0.99$ and lower case letters $P > 0.95$.

cient of variation was 3.6 % for 43 duplicate determinations. During a 6-month period (11 assays) the within-assay variation of the frozen pool was 2.1 % of a mean of 383.8 μkat/l (1.1—4.4 %). This pool was analysed 6—16 times in each assay during this period. The between-assay variation calculated on one randomly chosen value from each of these 11 assays was 7.5 % of a mean of 384 μkat/l.

Experiment 2

The results of determination of GSH-Px in erythrocytes from blood preserved with different concentrations of heparin are shown in Table 2. Storage gave a highly significant decrease in the measured activities. The high concentrations of 1000 and 1250 IU/ml gave the highest activities ($P > 0.95$) in samples immediately frozen for analysis.

DISCUSSION

The variability of the GSH-Px determination within an assay was low, below 5 %, measured both by the duplicate determinations of many samples and the repeated analyses of the reserve pool of cow blood. The between-assay variations were somewhat higher but acceptable. The frozen cow blood pool remained stable for the 6-month period tested and proved to be a good internal standard. By using successively new batches of blood it should be possible to use such samples as controls in this analysis.

The present results show that there is no inhibitory effect of heparin, even of high concentrations, on the determination of GSH-Px in blood from cows, goats and horses. On the contrary, higher readings were obtained in both experiments with high concentrations of heparin. This is contrary to the results of *Günzler et al.* (1974) who stated that heparin is a strong inhibitor of GSH-Px activity and that EDTA is the preferable anticoagulant. The reason for these differences in results could be due to the fact that *Günzler et al.* used a purified bovine enzyme in their experiments. It has been stated by *Mills* (1959) that purification of the enzyme results in a markedly increased lability.

As with most other enzymes, GSH-Px is affected by storage, especially at room temperature. There are, however, great dif-

ferences between species regarding the effect of storage. In the present study, horse blood appears to be the most sensitive to storage (Table 1). The highly significant decrease in GSH-Px activity shown in Table 2 is difficult to compare with the results in Table 1 because of the different concentrations of heparin used. The effect, although statistically significant, does not invalidate the analysis of samples sent by normal mail to the analyzing laboratory. Such results would, nevertheless, give an indication of a possible selenium deficiency. According to the present results the anticoagulant of choice would be heparin in concentrations normally used in blood samples.

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SAMMANFATTNING

Effekt av olika antikoagulantia på bestämning av glutathionperoxidas i erytrocyter.

Glutationperoxidas (GSH-Px) bestämdes i helblod från kor, getter och hästar. Cuminhydroperoxid användes som substrat vid bestämningarna som utfördes i en LKB 8600 Rection Rate Analyzed. Heparin var det mest lämpliga antikoagulantium som testades. De högsta aktiviteterna av enzymet uppmättes då höga halter av heparin fanns i blodprovet (1000 och 1250 IE/ml blod). Natriumfluorid men speciellt EDTA och natriumcitrat gav lägre enzymaktiviteter vid samtidig bestämning på samma blodprov.

Förvaring av blodproverna i rumstemperatur (20°C) eller i kylskåp (5°C) under 3 dagar gav signifikant lägre enzymaktiviteter. Detta var speciellt tydligt i hästblod.

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