STUDY ON GLUTATHIONE S-TRANSFERASE INHIBITION ASSAY BY TRICLABENDAZOLE. III: NEMATODIRUS PARASITE AND SHEEP LIVER TISSUE

A. Farahnak[1*], M. Anvar-Seyyedi [1] and M. Jalali [2]

1) Department of Parasitology and Mycology, School of Public Health, Medical Sciences/ University of Tehran, Tehran, Iran

2) Department of Nutrition and Biochemistry, School of Public Health, Medical Sciences/ University of Tehran, Tehran, Iran

Abstract- The most important and widely prevalent nematodes of sheep are the trichostrongyle group parasites, including nematodirus parasite. Accidental infection of man by nematodirus has been reported in Iran. Glutathione S-Transferase enzymes (GSTs) are detoxification enzymes in parasites such as nematodirus. Therefore, GST enzymes of these parasites could be a target for evaluation of drugs effect as triclabendazole ($C_{14}H_9CL_3N_2OS$). For this reason, GST enzymes were purified from nematodirus parasite and sheep liver tissue by glutathione affinity chromatography and prepared their SDS-PAGE banding pattern for GST fraction separation. GST enzymes specific activity levels are also assayed in the whole extract and purified solutions with reduced glutathione (GSH) and 1-chloro-2, 4-dinitrobenzen (CDNB) secondary substrate. Finally, GST inhibition assay was investigated in the solutions by powder and bolus forms of triclabendazole. The level of GST specific activity in purified solutions was detected 9.86 µmol / min/ mg protein for nematodirus parasite and 37.84 µmol/ min/ mg protein for liver tissue. Comparison of the effect of powder and bolus of triclabendazole on solutions revealed inhibition concentration (IC_{50}) 5.54 and 6.01 µg/ml for nematodirus GST and 8.65 and 9.70 µg/ml for liver tissue GST, respectively. These findings revealed the possibility of isolation and inhibition of nematodirus GST by triclabendazole, and more tolerance of liver tissue than parasite against this drug in vitro situation.

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Key words: Glutathione S-transferase, nematodirus, triclabendazole, parasite, nematode, helminth

INTRODUCTION

Trichostrongyle group parasites (*Nematodirus*, Haemonchus, Ostertagia, Trichostrongylus, Mecistocirrus, Cooperia, Oesophagostomum and Bunostomum) are the most important and widely prevalent nematodes parasites. In the recent years, nematodirus parasite has been reported from Iran (1).

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*** Corresponding Author:**
Ali Farahnak, Department of Parasitology and Mycology, School of Public Health, Medical Sciences/University of Tehran, Tehran, Iran
Tel: +98 21 88951583, Fax: +98 21 66462267
E-mail: farahnak@ tums.ac.ir

Accidental ingestion of infective stage in man could be happened with contaminated herbage (2).

GST enzymes of helminth parasites may protect against exogenous free radical damage or xenobiotics as a result of immune effector mechanisms from the host directed at the parasite (3, 4). Glutathione transferase activity has been detected in cestodes, digeneas and nematodes. Significantly higher activity has been found in intestinal cestodes and digeneas, compared with parasitic nematodes (5). GSTs isoenzymes of sheep liver could provide useful biomarkers for monitoring environmental pollution (6). Previous studies have been revealed

GST inhibition of *Fasciola hepatica* by hexachlorophene and characterization of purified GST by 2 - dimension electrophoresis (7, 8, 9). In addition, GST inhibition has been described in the cytosol of protoscoleces of hydatid cysts (Larva stage of *Echinococcus granulosus* parasite), *Fasciola hepatica* and *Fasciola gigantica* by triclabendazole from sheep in Iran (10, 11).

In the present study, the authors have reported the isolation, SDS-PAGE banding pattern, specific activity levels and specific activity inhibition assay of purified nematodirus GST and sheep liver tissue GST by triclabendazole.

MATERIALS AND METHODS

Nematodirus Parasite and Sheep liver Tissue Extract Solutions Preparation

Nematodirus parasites and liver tissue samples were obtained from sheep slaughtered at a local abattoir (Puryaye vali, Tehran, Iran). They were washed 3-4 times with washing buffer and homogenized in homogenizing buffer [10 mM EDTA, 2 mM PMSF, 0.15 M NaCL, 50 mM Tris (PH 7.5) containing 0.5% V/V Triton X-100 (Sigma)] by a glass homogenizer. Then suspension was centrifuged (12000g for 30 min at 4°C) and supernatant was stored at -80°C (10, 11).

Purified GSTs Solutions Preparation

GST enzymes were purified from nematodirus parasites and sheep liver extract solutions as enzyme pool by a glutathione-affinity matrix [glutathione-agarose (Sigma, Saint Louis, Missouri USA)] using by wash-batch method. For this purpose, Two hundred µl of glutathione- agarose gel (14 mg of powder in 200 µl of dionized water) was prepared in microtube Eppendorf. (12). Subsequently, 750 µl (2-3 mg protein) of purified solution was mixed with the gel for 30 min at 4°C and centrifuged at high speed (1000 g) for 10 sec in a bench microcentrifuge at 4°C. The supernatant was removed and the gel matrix washed with PBS-T (PBS 10 mM, pH 7.4 containing 50 mM NaCl and 1% Triton X-100) by centrifuge at high speed for 10 sec at 4°C. Finally, bound GST enzymes were eluted by washing the gels with elution buffer (50 mM Tris-HCL pH 9.6 buffer containing 5 mM reduced glutathione) and then centrifuged at high speed for 10 sec at 4°C and accumulated the supernatant into the clean tubes and stored at- 80°C (13).

Protein Assay of Solutions

The amount of protein in the extract solutions and purified solutions of nematodirus parasite and sheep liver were estimated by the method of Bradford using purified bovine serum albumin (1mg/ml) as the standard (14).

SDS-PAGE Pattern of Solutions

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was conducted as has been reported previously (15). Briefly, about 20 µl of extract and purified solutions from sheep liver tissue and nematodirus parasite were mixed with sample buffer and were run on 12.5% acrylamide gels. Obtained protein bands were stained with Coomassie blue R-250.

GST Specific Activity Assay of Solutions

GST activity was assayed spectrophotometrically at 30°C with 1-chloro-2, 4-dinitrobenzene (CDNB) as the standard second substrate and reduced glutathione (GSH) (10,11). This was done by watching an increase in absorbance at 340 nm. For this purpose, switch on spectrophotometer (CECIL 9000) and set water bath temperature to 30°C. Whole extract and purified solutions were removed from -80°C freezer and allowed to thaw on ice. CDNB 100 mM from 4°C and GSH 100 mM from -20°C freezer were removed and allowed to thaw at room temperature, when thawed, incubated at 30°C in water bath. For each assay, one ml of assay cocktail (980 µl PBS pH 6.5, 10 µl of 100 mM CDNB and 10 µl of 100 mM GSH) was prepared, and then removed 100 µl of cocktail and its remaining placed into 1.5 ml plastic cuvet. Distilled water was used as zero and 100 µl PBS was added to 900 µl of cocktail in the blank cuvet and absorbance was measured at 340 nm for 5 min. 100 µl of sample was added to 900 µl cocktail in the test cuvet and mixed and measured absorbance at 340 nm for 5 min. For calculation, the Δ340/min of blank

reaction was subtracted from the Δ340/min of each sample reaction. The molar extinction of CDNB is 0.0096 μM^{-1}/cm (16).

Effect of Triclabendazole on GST Activity in the whole extract and purified solutions

In the present study, GST inhibition enzyme assay was investigated in the extract and purified solutions of nematodirus and sheep liver by powder and bolus forms of triclabendazole (RAZAK Co).One mM triclabendazole solution was prepared by 3.59 mg of powder and 10.48 mg of bolus, each in 10 ml of ethanol.

Inhibition of GST specific activity was measured as a IC_{50}, which is defined as the concentration of antihelminth at which 50% of enzyme specific activity is inhibited. This was determinated by measuring GST specific activity using reduced glutathione and CDNB in the present of different concentration of powder and bolus triclabendazole solutions (10, 11).

Statistical analysis

Inhibition concentration of triclabendazole (IC 50%), bolus and powder, for parasite and liver tissue GST enzymes were obtained by regression analysis and compared two groups data for confidence intervals by using SPSS software.

RESULTS

SDS-PAGE banding pattern of GST enzymes in solutions are shown in Fig. 1. In this figure whole extract and purified solutions of sheep liver tissue show more band protein than nematodirus parasite. The level of GST activity and GST specific activity in solutions were detected, and the results presented in Table 1. The results of GST enzyme specific activity levels of extract and purified solutions from sheep liver tissue and nematodirus parasite

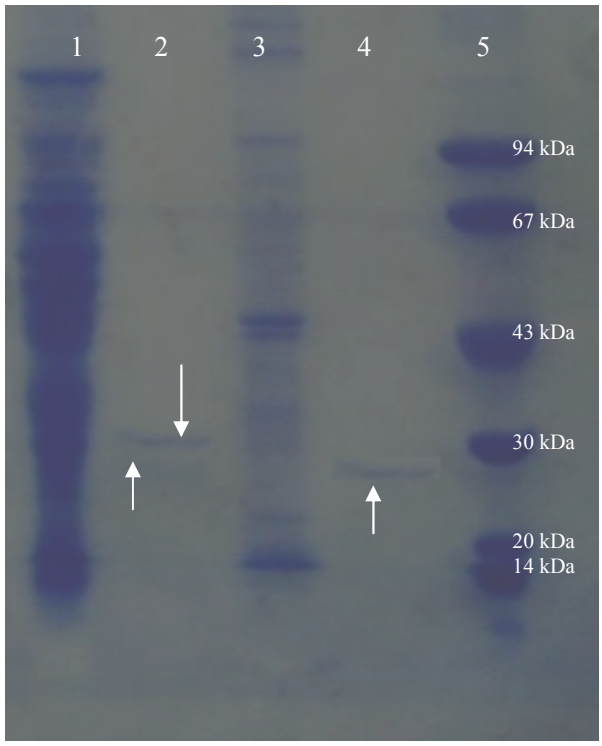

Fig. 1. SDS-PAGE pattern of extract and purified GST enzymes solutions of sheep liver tissue (Lane 1, 2), nematodirus parasite (lane 3, 4) and standard marker (lane 5).

demonstrate the liver GST activity in both cases were greater than parasite. The effects of triclabendazole (powder and bolus) on GST specific activity of extract and purified solutions were determined and the results are shown in Figs 2-5. The results presented in figs 2-5 show GST remaining specific activity based on triclabendazole concentration in the extract and purified solutions of sheep liver tissue in comparison with parasites not reach to zero and it is a good indicator for treatment of this parasitic disease in sheep. Finally, the inhibitor concentration for remaining specific activity of samples GST was calculated graphically and is presented in Table 2. These findings demonstrate that powder in comparison with bolus form drug with less concentration cause reduction enzyme activity to 50% of their levels.

Table 1. GST activity and GST specific activity of extract and purified solutions from sheep liver tissue and nematodirus parasite

Enzyme activity		GSTs activity (U/ml)	GSTs specific activity (µmol/min/mg protein)
Homogenized solutions	Nematodirus parasite	0.055	3.14
	Liver tissue	14.75	446.97
Purified solutions	Nematodirus parasite	0.0098	9.86
	Liver tissue	0.17	37.84

Glutathione S-transferase inhibition assay

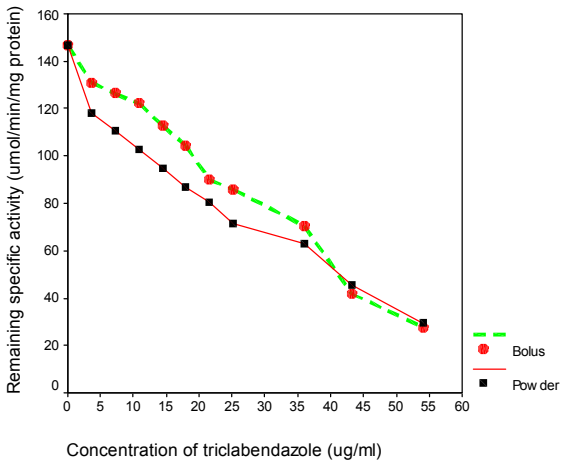

Fig. 2. GST remaining specific activity based on triclabendazole concentration in the extract solution of sheep liver tissue.

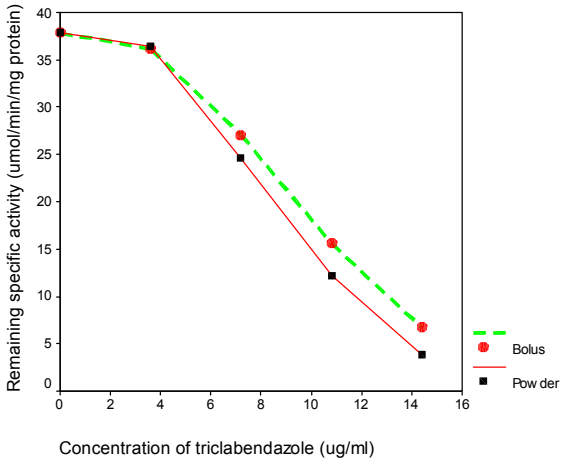

Fig. 3. GST remaining specific activity based on triclabendazole concentration in the purified solution of sheep liver tissue.

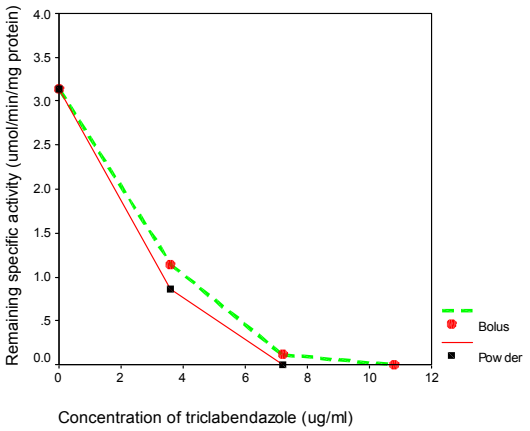

Fig. 4: GST remaining specific activity based on triclabendazole concentration in the extract solution of nematodirus parasite.

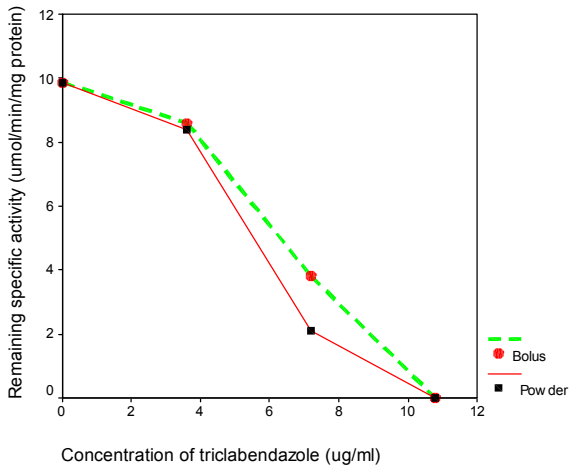

Fig. 5. GST remaining specific activity based on triclabendazole concentration in the purified solution of nematodirus parasite.

DISCUSSION

SDS-PAGE of the present study pattern was revealed one band protein with 26 kDa that showed purified GST enzymes in the purified solution of nematodirus parasite; however sheep liver tissue showed two bonds ranging 26-28 kDa, because some isoenzymes of GST are present in the liver. The results showed that GST activity in liver solution was grater than nematodirus solution, because there were more enzymes than parasite that could affect on the reaction. In parasitic helminthes, the highest glutathione transferase activities are found in cestodes and digeneans, with lower activities in nematodes, including nematodirus parasite (3).

Detoxification is probably achieved through the glutathione S-transferase and it is possible that resistant strains express a greater amount of this enzyme when treated with the drug compared with susceptible strains. Inhibition of GST enzyme activity from nematode has been shown by extract of

Table 2. Inhibition concentrations of 50% of GST specific activity (IC_{50}) of nematodirus parasite and sheep liver tissue by triclabendazole (powder and bolus)

Solutions		IC_{50}	
		Bolus	Powder
Nematodirus parasite	Homogenized	3.77	3.33
	Purified	6.01	5.54
Liver tissue	Homogenized	49.66	40.95
	Purified	9.7	8.65

Nigerian plants (17). Comparison of the effect of triclabendazole on GST specific activity revealed that IC_{50} for bolus was greater than powder in both samples. This phenomenon may be due to this fact that the amount of triclabendazole itself in bolus is less than that of issued on its box or may be due to existence of supplementary materials in bolus, for protecting of drug from the digestive enzymes, so the effectiveness of the powder form to bolus is a reasonable fact. Comparison of the effect of triclabendazole bolus and powder base on regression analysis and confidence intervals by SPSS software revealed that activities of both GST enzymes were suppressed but the difference between inhibition assays was not significant.

In brief, these findings show possibility of isolation and inhibition of nematodirus GST by triclabendazole in vitro situation and more tolerance of liver tissue than parasite against this drug in vitro situation.

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Conflict of interests

The authors declare that they have no competing interests.

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