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Purification and Characterization of Glucose 6-phosphate Dehydrogenase from Sheep Erythrocytes and Inhibitory Effects of some Antibiotics on Enzyme Activity

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Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49; G6PD) was purified from sheep erythrocytes, using a simple and rapid method. The purification consisted of three steps; preparation of haemolysate, ammonium sulphate fractionation and 2′, 5′-ADP Sepharose 4B affinity chromatography. The enzyme was obtained with a yield of 37.1% and had a specific activity of 4.64 U/mg proteins. Optimal pH, stable pH, molecular weight, and K_M and V_{max} values for $NADP^+$ and glucose 6-phosphate (G6-P) substrates were also determined for the enzyme.

The overall purification was about 1,189-fold. A temperature of +4°C was maintained during the purification process. In order to control the purification of the enzyme SDS polyacrylamide gel electrophoresis (SDS-PAGE) was done in 4% and 10% acrylamide concentration for stacking and running gel, respectively. SDS-PAGE showed a single band for enzyme. Enzymatic activity was spectrophotometrically measured according to Beutler's method at 340 nm.

In addition, *in vitro* effects of gentamicin sulphate, penicillin G potassium, amicasin on sheep red blood cell G6PD enzyme activity were investigated. These antibiotics showed inhibitory effects on enzyme activity. I_{50} values were determined from Activity%–[Drug] graphs and K_i values and the type of inhibition (noncompetitive) were determined by means of Lineweaver–Burk graphs.

Keywords: Glucose 6-phosphate dehydrogenase; Drug; Sheep; Erythrocyte

INTRODUCTION

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49; G6PD) is present in almost all mammalian tissues, in plants, and microorganisms. The enzyme is located in the cytosol and mitochondria of animal cells and in the cytosol and chloroplasts of green plants.[1–4] It is widespread in all tissues and blood cells and is a housekeeping enzyme.[5]

G6PD is the key enzyme, which catalyzes the first step of the hexose monophosphate shunt (HMPS) pathway.[2,6] The principal source of cytoplasmic NADPH in many cells is the hexose monophosphate pathway and specifically the two deydrogenase's, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. These reactions appear to be necessary for maintenance of cellular integrity. NADPH production is decreased in G6PD deficiency. In mammalian red cells, the production of hydrogen peroxide by endogenous mechanisms and environmental substances is offset by a mechanism requiring glutathione and, in turn, NADPH.[7,8] The reduced glutathione form contains a tripeptide which has a free thiol group. This form acts as an antioxidant and keeps the cystein residues of hemoglobin and proteins in the erythrocyte reduced. Normally, the ratio of reduced glutathione to oxided glutathione is approximately 500. Reduced glutathione also plays a role in some detoxification reaction by reducing inorganic and organic peroxide.[9,10]

G6PD was first isolated from human erythrocytes by Yoshida.[11] In the following years, the enzyme was purified from ion-exchange materials by using the natural substrates, Glucose 6 phosphate (G6-P) and $NADP^+$, resulting in a high degree of purification.[1,11] Affinity chromatography (2′,5′-ADP Sepharose 4B)

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used first by De Flora and co-workers is the common technique for human G6PD purification.[1] Purification and characterization by 2′,5′-ADP Sepharose 4B affinity chromatography of sheep erythrocyte G6PD has not been described in the literature. Some modifications to this technique have been made and by our procedure described here, the enzyme can be purified within a very short time and in good yield.

The present study documents a fast, simple purification procedure for sheep erythrocyte G6PD and examines the kinetic behaviour of the enzyme. The effects of some drugs on enzyme activity *in vitro* have been examined since these drugs are commonly used in the treatment of many sheep diseases.

MATERIALS AND METHODS

Materials

2′,5′-ADP Sepharose 4B was purchased from Pharmacia. $NADP^+$; glucose 6-phosphate and protein assay reagent were purchased Sigma Chem. Co. All other chemicals used were analytical grade and purchased from either Sigma or Merck. Gentamicin sulphate, penicillin G potassium, amicasin were obtained from the Hospital of Medical Science Faculty (Erzurum, Turkey).

Preparation of the Haemolysate

Fresh sheep blood collected in tubes with EDTA (5 mM) was centrifuged at 2500 × g for 15 min and the plasma and leucocyte coat were removed by drip. The packed red cells were washed with KCl solution (0.16 M) three times. The samples were centrifuged at 2,500 × g each time and supernatants were removed. One-volume erythrocytes was haemolysed with 5 volumes of ice-cold water and centrifuged at 4°C, 10000 × g for 30 min to remove the ghosts and intact cells.[12]

Ammonium Sulfate Fractionation and Dialysis

The haemolysate was subjected to orderly precipitation with ammonium sulphate (10–20%, 20–30%, 30–40%, 40–50%, 50–60%, 60–70% and 70–80%). Ammonium sulphate was slowly added to the haemolysate for completely dissolution. This mixture was centrifuged at 10,000 × g for 15 min and the precipitate was dissolved in 50 mM of phosphate buffer (pH 7.0). For each respective precipitation, the enzyme activity was determined both in the supernatant and in the precipitate. The enzyme was observed to precipitate at 50–60% saturation. It was then dialysed at 4°C in 50 mM K-acetate/5 mM K-phosphate buffer (pH 7.0) for 2 h with two changes of buffer.[12]

2′,5′-ADP Sepharose 4B Affinity Chromatography

Dried 2′,5′-ADP Sepharose 4B gel (2 g) was used for a 10 mL column volume. The gel was washed with 400 mL distilled water, to remove foreign bodies and air, and then suspended in 0.1 M K-acetate/0.1 M K-phosphate buffer (pH 6.0) and packed in a small column (1 × 10 cm) and equilibrated with the same buffer. The gel was washed with equilibration buffer. The flow rates for washing and equilibration were adjusted by peristaltic pump to 50 mL/h. The dialysed sample obtained previously was loaded onto the 2′, 5′-ADP Sepharose 4B affinity column and the flow rate was adjusted to 20 mL/h. The column was then, sequentially washed with 25 mL of 0.1 M K-acetate + 0.1 M K-phosphate, (pH 6.0) and 25 mL 0.1 M K-acetate + 0.1 M K-phosphate (pH 7.85). The latter washing was continued until the final absorbance difference became 0.05. Elution was carried out with 80 mM K–phosphate + 80 mM KCl + 0.5 mM $NADP^+$ + 10 mM EDTA (pH 7.85). The enzyme activity was measured in the final fractions and the activity-containing tubes were pooled. All of the procedures were performed at 4°C.[11–13]

Activity Determination

Enzymatic activity was measured by Beutler's method.[14] One enzyme unit was defined as the amount of enzyme reducing 1 μmol $NADP^+$ per 1 min.

Protein Determination

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method, with bovine serum albumin as standard.[15]

Optimal pH Determination

For the optimal pH determination, the enzyme activity was measured in 1 M Tris–HCl and phosphate buffers over the pH ranges 7.2–8.9 and 4.9–8.0, respectively.

Stable pH Determination

For this purpose, enzyme activity was determined in 1 M Tris–HCl buffer at pH 7.5, 8.0, 8.5 and 8.9, and in 1 M phosphate buffer at pH 5.0, 6.0, 7.0, and 8.0. In each experiment, equal volumes of buffer and enzyme solutions were mixed and kept refrigerated (+4°C). Activity determinations were made every 8 h over a period of for 24 h.

Molecular Weight Determination

By Sephadex G-200 Gel Filtration Chromatography

The molecular weight of the enzyme was determined using Andrew's method.[16] The enzyme-containing tube was first determined. The void volume was observed with Blue Dextrane 2,000 kDa. Horse heart cytochrome C (12.4 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), yeast alcohol dehydrogenase (150 kDa) and sweet potato β-amylase (200 kDa) were used as standards (Sigma: MW-GF-200).

SDS-PAGE

The subunit determination was made by SDS-PAGE.[17] Rabbit heart creatine phosphokinase (81 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa) and horse heart cytochrome C (12.4 kDa) were used as standards.

Kinetic Studies

In order to obtain K_M and V_{max} values separately for $NADP^+$ and G6-P substrates, at optimum pH and 25°C the enzyme activity was measured at five different substrate cuvette concentrations for $NADP^+$ (1×10^{-5}, 5×10^{-5}, 1×10^{-4}, 2×10^{-4} and 5×10^{-4} mM) with a constant concentration of G6-P (6.0 mM), and for G6-P (6.0, 12, 24, 30 and 36 mM) with a fixed $NADP^+$ (2 mM) concentration. K_M and V_{max} values were determined by means of Lineweaver–Burk graphs.

In addition, the effects of gentamicin sulphate, penicillin G potassium, and amicasin antibiotics were investigated for four different cuvette concentrations. Enzyme activities were measured for gentamicin sulphate (0.335 mM, 2.25 mM, 3.42 mM, 5 mM, 7.75 mM 12.50 mM and 16.70 mM), penicillin G potassium (3.22 mM, 9.66 mM, 16.10 mM, 22.54 mM and 32.20 mM) and amicasin (1.70 mM, 8.50 mM, 34 mM, 68 mM and 102 mM, and 119 mM) at cuvette concentrations. Control cuvette activity in the absence of drug was taken as 100%. For each drug an Activity% − [Drug] graph was drawn. Drug concentrations that produce 50% inhibition (I_{50}) were calculated from graphs.

To determine K_i values, three different inhibitor concentrations were tested; gentamicin sulphate: 3.35, 10.08, and 16.80 mM; penicillin G potassium: 4.83, 11.30, and 22.57 mM; amicasin: 8.54, 21.20, and 34.16 mM. In these experiments, G6-P was used as substrate at five different concentrations (0.024, 0.048, 0.072, 0.12 and 0.19 mM for gentamicin sulphate and penicillin G potassium; 0.14, 0.21, 0.36, 0.43 and 0.59 mM for amicasin). The Lineweaver–Burk curves obtained were used for the determination of K_i and the inhibitor type.[18] Analysis of data obtained was made by **t**, test and are given as $\bar{X} \pm SD$.

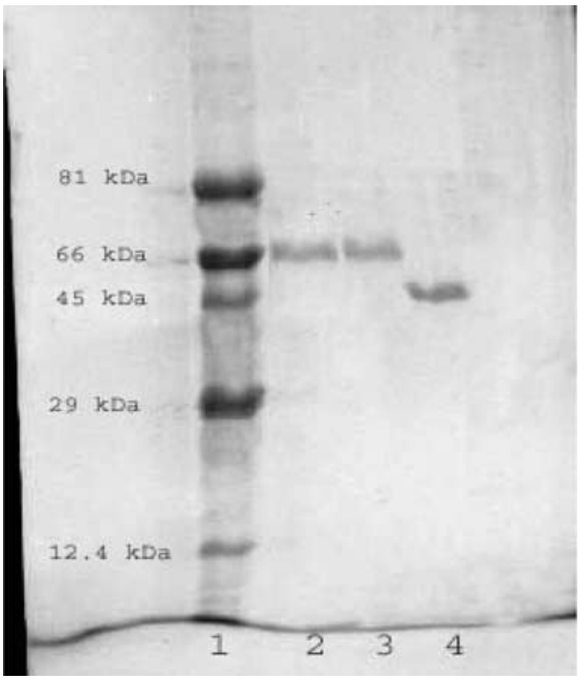

FIGURE 1 SDS-polyacrylamide gel electrophoresis of G6PD purified by affinity gel. Lane 1: Standard proteins; Lane 2: G6PD.

RESULTS

Table I shows a purification characterized by a specific activity of 4.64 EU/mg protein, a yield of 37.1% and a purification coefficient of 1,189. Figure 1 shows the SDS-PAGE made for determining the purity and molecular weight of the enzyme. For the standard proteins and G6PD, R_f values were calculated. The R_f − Log MW graph (Figure 2) was obtained according to Laemmli's procedure,[17] and

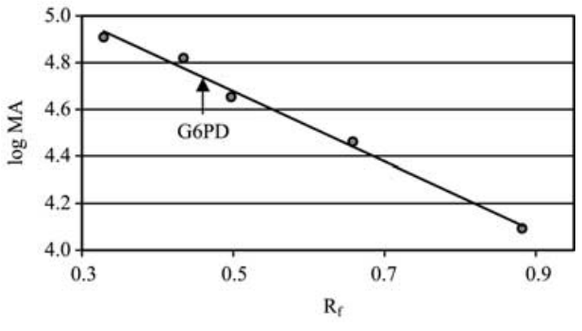

FIGURE 2 Standard R_f − Log MW graph of G6PD using SDS-PAGE (Standards: Rabbit heart creatine phosphokinase (81 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa) and horse hearth cytochrome C (12.4 kDa)).

TABLE I Purification scheme for glucose 6-phosphate dehydrogenase from sheep erythrocytes

Purification step	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Haemolysate	0.064	176	16.2	2837.12	11.264	0.0040	100	1
Ammonium sulfate precipitation (50–60)%	0.088	60	5.91	354.6	5.28	0.015	46.87	3.75
2′, 5′-ADP Sepharose 4B affinity chromatography	0.209	20	0.045	0.90	4.18	4.64	37.1	1.189

shows a molecular weight of 66,880 Da for G6PD. The molecular weight of the enzyme was also determined by gel filtration chromatography. The K_{av} − log MW graph (Figure 3), shows a molecular weight of 119,662 Da for G6PD. The Optimal pH of G6PD was determined as 8.0 using 1 M Tris–HCl (Figure 4). The stable pH of the enzyme was 8.5 in Tris–HCl (Figure 5).

The Lineweaver–Burk graphs are shown in Figures 6a and 6b, which were constructed for G6-P and $NADP^+$ respectively. A K_M of 0.036 ± 0.002 mM and a V_{max} of 0.059 ± 0.002 EU/ml were obtained for G6-P, and values of $1.23 \times 10^{-5} \pm 1.52 \times 10^{-7}$ mM and 0.012 ± 0.001 EU/ml for $NADP^+$. I_{50} values were 10.01 mM, 12.83 mM, and 41.88 mM for gentamicin sulphate, penicillin G potassium and amicasin, respectively (Figure 7, Table II).

K_i values of 0.308 ± 0.12 mM, 0.748 ± 0.17 mM and 12.253 ± 2.76 mM were found for gentamicin sulphate, penicillin G potassium and amicasin, respectively (Figure 8, Table II).

DISCUSSION

The importance of G6PD in metabolism has been well known for many years. We consider that an easier purification method of this enzyme makes it possible that investigations on this subject can be more easily done.

The enzyme has been eluted from ion exchange material by using one of its ligands. De Flora *et al.* were the first to use 2′,5′-ADP Sepharose 4B for this purpose.[1] These investigators used three consecutive steps for purification: DEAE Sephadex, Phosphocellulose (P11), and affinity chromatography on 2′,5′-ADP Sepharose 4B. These three steps a take long time and thus result in a decline in enzyme activity during the procedure. For this reason, we omitted the first two steps. After the haemolysate was precipitated with ammonium sulphate, the sample was directly

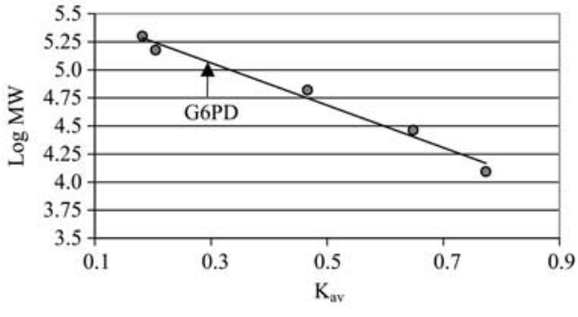

FIGURE 3 Standard K_{av} − log MW graph of G6PD using gel filtration, (Standards: Horse heart cytochrome C (12,400), bovine erythrocyte carbonic anhydrase (29,000), bovine serum albumin (66,000), yeast alcohol dehydrogenase (150,000) and sweet potato β-amylase (200,000)).

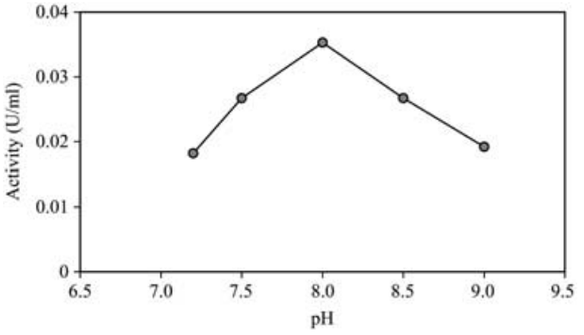

FIGURE 4 Activity-pH graph of G6PD.

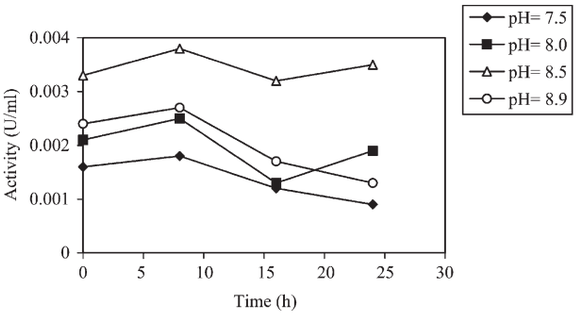

FIGURE 5 pH-stability graphs of G6PD in 1 M Tris–HCl buffer.

applied to a 2′,5′-ADP Sepharose 4B column. Moreover, we used 0.5 mM $NADP^+$ instead of 0.2 mM used by Ninfali *et al.* for elution, resulting in more a concentrated enzyme eluent.[12] Before affinity chromatography, so as to remove the impurities and to obtain a concentrated enzyme, ammonium sulphate precipitation (20–50%) was conducted. Due to the procedure used in this study, the enzyme having a specific activity of 4,64 EU/mg protein can be purified from 176 mL of whole blood within 5 or 6 h with a yield of 37% and 1,189 fold concentration and of high purity (Figure 1). In addition, the advantage of the purification method suggested in the study is that it is a less time-consuming than other methods and of low cost.

Another most important advantage of this modified method is that the 6-phosphogluconate dehydrogenase (6PGD) enzyme, the second step enzyme of the pentose phosphate pathway was removed during purification, which was confirmed by the fact that the purified enzyme solution did not contain 6PGD activity.

Optimal pH of G6PD has been determined as pH 8.0 using 1 M Tris–HCl (Figure 4) a result which was similar to that from previous studies.[14,19,20] The stable pH of the enzyme was pH 8.5 in 1 M Tris–HCl (Figure 5).

G6PD enzyme from different sources can form dimers, trimers, tetramers and hexamers of identical subunits. The minimum structure, necessary for catalysis, is the dimeric form.[1,21] The molecular weight of the G6PD from sheep erythrocytes was found to be 119,662 Da using Sephadex G-150 gel filtration chromatography and 66,800 Da using SDS-PAGE electrophoresis. Since the molecular weight determined by gel filtration chromatography was approximately twice that by SDS-PAGE electrophoresis then sheep erythrocyte active G6PD may be found in the dimer form. Similar results have been observed for the enzymes obtained from human erythrocytes (120 kDa; dimer), human liver (118 kDa; dimer) and rat erythrocytes (131 kDa; dimer).[22]

The K_M for $NADP^+$ is lower than that for G6-P, suggesting the higher affinity of G6PD to $NADP^+$ when compared with G6-P. K_M values are very similar to those obtained in rat cortex and liver,[23] in dog liver[20] and in human placenta.[24]

G6PD has a vital function in many kinds of tissues and plays an important role in their metabolism. Many chemicals at relatively low dosages affect the metabolism of biota by altering normal enzyme activity, particularly inhibition of a specific enzyme,[25] and the effects can be dramatic and systemic.[26] Some chemicals and drugs also inhibit

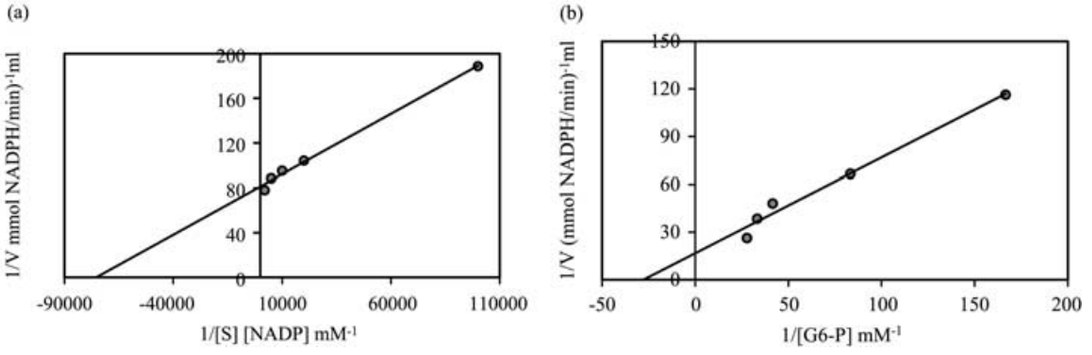

FIGURE 6 Lineweaver–Burk graphs in 5 different $NADP^+$ concentrations in constant G6-P concentration (6a), and in 5 different G6-P concentrations in constant $NADP^+$ concentration (6b).

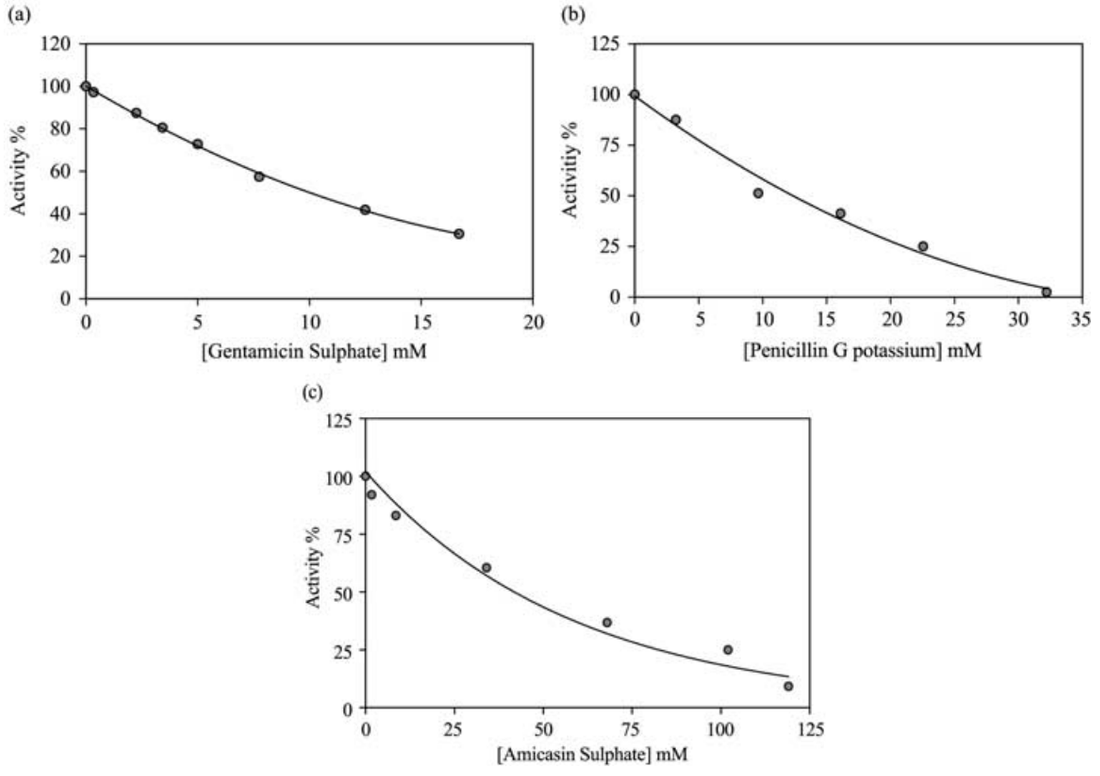

FIGURE 7 Activity% *vs* [Gentamicin sulphate] (7a), Activity % *vs* [Penicillin G potassium] (7b), and Activity% *vs* [Amicasin sulphate] (7c), regression analysis graphs for G6PD.

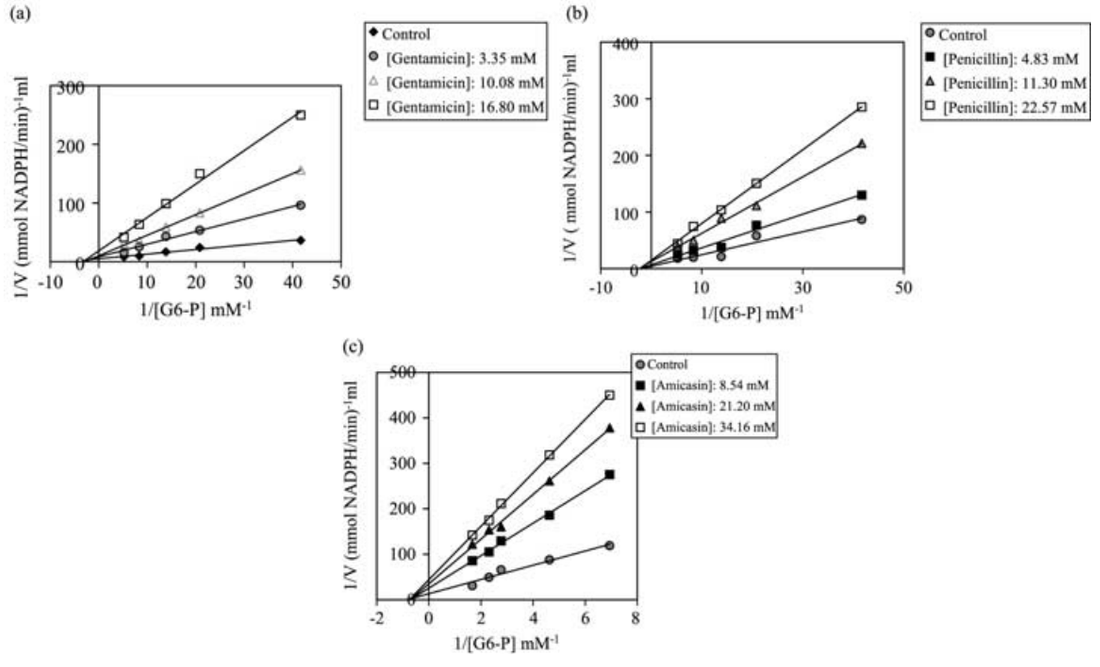

FIGURE 8 Lineweaver–Burk graph in different substrate (G6-P) concentrations for determination of K_i of gentamicin sulphate (8a), penicillin G potassium (8b) and amicasin sulphate (8c).

TABLE II I_{50} values and K_i constants for gentamicin sulphate, penicillin G potassium, amicasin sulphate

Drug	I_{50} value (mM)	Ki value (mM)	Inhibition type
Gentamicin sulphate	10.01	0.308 ± 0.12	Noncompetitive
Penicillin G potassium	12.83	0.748 ± 0.17	Noncompetitive
Amicasin sulphate	41.88	12.253 ± 2.76	Noncompetitive

G6PD enzyme activity. For example, sodium cefuroxime and sodium ceftizoxime inhibit the G6PD enzyme activity from human red cells.[27]

Gentamicin sulphate, penicillin G potassium and amicasin as antibiotics were chosen for investigation of their inhibitory effects on G6PD. In this study, both the K_i and I_{50} parameters of these antibiotics for G6PD were determined and the inhibition was noncompetitive (Figure 8). These antibiotics have shown inhibitory effects on G6PD enzyme activity but gentamicin sulphate is the most potent (Table II). Therefore, if these drugs are given to sheep, their dosages should be very well controlled ordered to prevent their side effect on red blood cells. For the same antibiotics, similar results have been observed for the enzymes obtained from human erythrocytes.[27]

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