THE EFFECTS OF ASPIRIN METABOLITES ON NADH-METHAEMOGLOBIN REDUCTASE (DIAPHORASE) ACTIVITY IN HUMAN ERYTHROCYTES

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SUMMARY : The present study was undertaken to know whether aspirin metabolites (sodium salicylate, Na-SA; sodium gentisate, Na-GA; sodium salicylate, Na-SA; sodium gentisate, Na-GA; sodium salicylurate, Na-SU) exert their reported toxic effects by inhibiting the enzyme 'NADH-Methaemoglobin (MHb) reductase (Diaphorase)' in normal adult human erythrocytes. The inhibition of the enzyme activity in normal haemolysates was observed to be highest with Na-SU, followed by Na-GA and Na-SA respectively (92%, 90% and 75% inhibition with 0.13 mM Na-SU, 50 mM Na-GA and 50 mM Na-SA respectively). The Linewearer-Burk plots from the kinetic studies (Vmax) with varying concentrations of the substrate 'NADH' indicated that the inhibition of the enzyme activity by all the three aspirin metabolites were of non-competitive type [Vmax(U)→NADH alone : 47.64 ± 4.56, NADH + 10 mM Na-SA : 24.39 ± 2.34, P<0.02; Vmax (U)→NADH→NADH alone : 16.67 ± 1.12, NADH + 0.002 mM Na-SU : 7.09 ± 1.05, P<0.01]. The replacement of 'MHb' by the dye '2,6-dichlorobenzene-indophenol' suggested that the inhibitory effects were due to direct interaction of the aspirin metabolites with the enzyme molecules. No evidence was also found that aspirin metabolites interact directly with NADH. It was, therefore, concluded that the aspirin metabolites (Na-SA, Na-GA, Na-SU) exert their reported toxic effects through inhibition of NADH- MHb reductase activity in a non-competitive manner probably by interacting at a region near the 'active site' causing allosteric (conformational) changes in the enzyme molecules. These conformational changes, in turn, inhibit the catalytic activity of the enzyme 'Diaphorase' in human erythrocytes leading to toxic manifestations.

Key Words : Aspirin, salicylate, gentisate, salicylurate, erythrocyte, NADH-Mathaemoglobin reductase, Km, Vm.

INTRODUCTION

Although aspirin (acetylsalicylic acid) is classified as a mild haemolytic agent, it has been reported on several occasions to give rise to severe haemolysis of erythrocytes even after small therapeutic doses (1, 2, 37). Most of the drugs cause haemolysis, especially in glucose-6-phosphate dehydrogenase (G6PD) deficient individuals, through oxidative damage by their metabolites rather than the parent compounds themselves (4- 6). We have suggested that aspirin metabolites (Salicylic acid, SA; Salicyluric acid, SU; Gentisic acid, GA) exert their toxic effects probably by acting at different sites of reduced glutathione (GSH) and hemoglobin (Hb) metabolic pathways (7, 8). This was based on our observations that while GA oxidised Hb to methemoglobin (MHb), SA and SU did not show appreciable oxidative effects on Hb (8). But, all the

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three aspirin metabolites exerted inhibition of MHb reduction significantly, although GA showed the highest effects (8). The formation of MHb (usually < 1.0%) and its reduction back to Hb is a normal physiological process and the main enzyme systems involved in this process are NADH-MHb reductase (Diaphorase) and NADPH-MHb reductase which is linked via NADH to Emden-Meyerhof pathway of glycolysis (9, 10). This has raised the question whether aspirin metabolites exert their toxic effects as reported (8) by acting as inhibitors of the enzyme NADH-MHb reductase. Several dehydrogenase and hexokinase enzymes were, however, reported to be inhibited by SA (11-13). The effects of aspirin metabolites (SA, SU and GA) an NADH-MHb reductase (Diaphorase) were, therefore, investigated in detail in normal erythrocyte hemolysates and the results are presented in this communication.

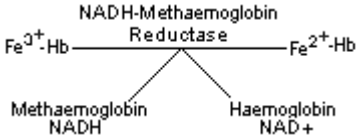
MATERIAL AND METHODS

Preparation of erythrocyte hemolysate

Two male adult volunteers were selected at random with normal G6PD activity. Venous blood was drawn from the antecubital veins in heparinised tubes and processed to prepare erythrocytes, free of leucocytes and platelets, to their original packed cell volume (PCV). The hemolysates were then prepared by adding 9 ml distilled water to 1 ml of the erythrocyte preparation. The lysates were allowed to stand at room temperature for 10 minutes and centrifuged at 1650 g to remove cell stroma. The supernatants were separated, hemoglobin contents were determined by cyanomet hemoglobin method (14) and G6PD activity was measured by using diagnostic kits from bio Meriux, France (15). The hemolystase jith normal G6PD activity were used in individual experiments for assaying NADH-MHb reductase activity.

Assay of NADH-MHb reductase (Diaphorase) activity

The enzyme activity was assayed in the presence or absence of aspirin metabolites according to the modified method of Worathumrong and Grimes (16). The assay (incubation) mixture of 2.6 ml was composed of the following: 1.0 ml reaction mixture [0.25 mM $K_3Fe(CN)_6$ + 0.05 mM Hb+ 1.25 mM EDTA + 0.01 mM Tris-HCl buffer, pH 5.5] + 0.1 ml hemolysate + 1.3 ml aspirin metabolite solution + 0.15 ml H_2O + 0.05 ml of 4.2 mM NADH. The reaction was started by the addition of NADH and the increase in absorbance was followed at 575 nm over the linear period of the reaction. From the hemolysate hemoglobin concentration in the incubation mixture and the change in optical density per minute at 575 nm (Δ/min), the enzyme activity was calculated. The amount of enzyme which catalyses the reduction of 1 n mole of Fe^{3+}-Hb/min/mg Hb was defined as 1 unit of NADH-MHb reductase activity. The overall reaction was represented by the following equation (16) :



Effects of various concentrations of aspirin metabolites on NADH-MHb reductase activity

The aspirin metabolites used in this experiment were sodium salicylate (Na-SA), sodium gentisate (Na-GA) and sodium salicylurate (Na-SU) at final concentrations (mM) as follows : Na-SA $\rightarrow$ 5, 10, 15, 25, 50; Na-GA$\rightarrow$ 5, 10, 15, 25, 50; Na-SU$\rightarrow$0.009, 0.018, 0.035, 0.07, 0.13. The reaction was initiated by adding 0.05 ml of 4.2 mM NADH so that the optimal (Final) NADH concentration of 0.08 mM was obtained in the total assay volume of 2.6 ml containing the hemolysate as the source of enzyme. The enzyme activities were calculated from the linear part of the increase in absorbance at 575 nm as described in the preceding section (16).

Effects of various concentrations of NADH on the NADH-MHb reductase activity in the presence of aspirin metabolites

The same assay system was used as described in the preceding experiment. The reaction was started by adding 0.05 ml aliquots of NADH solutions so that 0.20, 0.04, 0.08, 0.12, 0.16 and 0.20 Mm final concentrations were obtained in a total assay volume of 2.6 ml in the absence or presence of aspirin metabolites (Na-SA : 10 mM; Na-GA : 5 mM and 10 mM; Na-SU : 0.02 mM and 0.05 mM). The enzyme activities were obtained from the linear part of the absorbance at 575 nm as described in preceding experiments (16).

Direct effects of aspirin metabolites on NADH-MHb reductase activity

In this experiment the natural substrate 'MHb' was substituted with a blue dye '2,6-dichlorobenzene-indophenol (DCI)'. The enzyme assays in the hemolysates were carried out according to the method of Scott (17) as modified by Worathumrong and Grimes (16). The dye 'DCI' (final conc. : 0.015 mg%) was reduced by NADH (final conc. : 0.028 mM) to a colorless compound at a rapid rate. Thus any effect imposed on the rate of reduction of DCI by aspirin metabolites can be assumed not to be upon MHb, but upon the enzyme 'NADH-MHb reductase' itself or upon NADH. The reduction of DCI was followed at 600 nm with or without Na-

Table 1:The effects of various concentrations of aspirin metabolites on NADH-MHb reductase activity in the presence of 0.08 mM NADH.

Aspirin metabolites (mM)	NADH-MHb reductase activity (U)*	Percentage inhibition (%)
Na-SA		
0 (Control)	13.62 ± 0.27	0.0
5	17.73 ± 0.33	14.0
10	11.05 ± 0.21	18.9
15	9.91 ± 0.16	27.3
25	5.85 ± 0.10	57.1
50	3.39 ± 0.05	75.0
Na-GA		
0 (Control)	12.88 ± 0.25	0.0
5	9.17 ± 0.18	29.0
10	5.30 ± 0.11	58.8
15	4.31 ± 0.08	66.5
25	3.02 ± 0.05	76.5
50	1.34 ± 0.02	90.0
Na-SU		
0.0 (Control)	15.85 ± 0.31	0.0
0.009	15.10 ± 0.29	5.0
0.018	11.39 ± 0.23	28.1
0.035	7.43 ± 0.13	53.1
0.070	3.72 ± 0.05	76.6
0.130	1.13 ± 0.02	92.0

*U: Enzyme activity in units; each value is the mean ± SD of 4 observations made in duplicate with 2 hemolysate preparations.

Figure 1:The effects of various concentrations of NADH (0.02-0.20 mM) on Diaphorase activity in the absence or presence of 10 mM Na-SA in human erythrocytes.

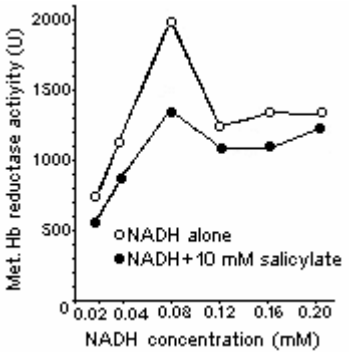


Table 2: The parameters (mean values) for Lineweaver-Burk plot derived from the initial linear phase of the curves, i.e. from Figures 1, 2 and 3.

	* Parameters for Linewear-Burk plot			
	S (mM)	I/S	V (U)**	I/V
NADH alone :	0.02 0.04 0.08	50.0 25.0 12.5	7.43 ± 0.71 11.89 ± 0.89 20.31 ± 2.03	$1.35x10^{-1}$ $0.84x10^{-1}$ $0.49x10^{-1}$
NADH+10 mM Na-SA :	0.02 0.04 0.08	50.0 25.0 12.5	5.70 ± 0.51 8.85 ± 0.72 13.87 ± 1.02	$1.75x10^{-1}$ $1.12x10^{-1}$ $0.72x10^{-1}$
NADH alone :	0.02 0.04 0.08	50.0 25.0 12.5	5.75 ± 0.50 8.20 ± 0.75 12.90 ± 0.87	$1.73x10^{-1}$ $1.22x10^{-1}$ $0.78x10^{-1}$
NADH+5 Mm Na-GA :	0.02 0.04 0.08	50.0 25.0 12.5	3.91 ± 0.30 4.95 ± 0.41 7.01 ± 0.67	$2.56x10^{-1}$ $2.02x10^{-1}$ $1.43x10^{-1}$
NADH alone :	0.02 0.04 0.08	50.0 25.0 12.5	8.52 ± 0.82 9.37 ± 0.91 11.01 ± 0.95	$1.17x10^{-1}$ $1.06x10^{-1}$ $0.91x10^{-1}$
NADH+0.02 mM Na-SU :	0.02 0.04 0.08	50.0 25.0 12.5	5.00 ± 0.45 5.74 ± 0.51 7.08 ± 0.65	$2.00x10^{-1}$ $1.74x10^{-1}$ $1.41x10^{-1}$

*S : Substrate (NADH); V: Enzyme activity in units;
**V (U) : Each value is the mean ± SD of 4 observations made in duplicate with 2 hemolysate preparations.

SA, Na-GA and Na-SU at a final concentration of 50 mM, 10 mM and 0.8 mM respectively in EDTA (0.3 M)-Tris (0.16 M) buffer (pH-7.5).

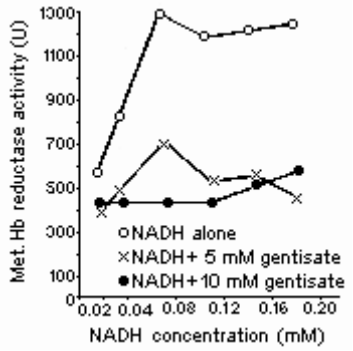
Direct effects of aspirin metabolites on NADH

This experiment was done to exclude the possibility of direct effects of aspirin metabolites on NADH by adopting the procedure described in the preceding experiment (16, 17). The optical density was followed at 366 nm in phosphate buffer (0.1 M, pH-6.9) with 0.036 mM NADH in the presence or absence of aspirin metabolites. The final concentrations of Na-GA and Na-SU were 50 mM, 10 mM and 0.8 mM respectively.

RESULTS

Table 1 shows the inhibitory effects of different concentrations of Na-SA (5-50 mM), Na-SU (0.009-0.13 mM) and Na-GA (5-50 mM) on NADH -MHb reductase activity in hemolysates prepared from the 2 male volunteers with normal G6PD activity. The inhibition of the enzyme activity was about 14% at 5 mM rising to 75% with 50 mM Na-SA,

Figure 2: The effects of various concentrations of NADH (0.02-0.20 mM) on Diaphorase activity in the absence or presence of Na-GA (5 mM and 10 mM) in human erythrocytes.



while it was about 29% and 90% with 5 mM and 50 mM Na-GA respectively. Na-SU was found to be most inhibitory towards NADH-MHb reductase activity being 5% and 92% with 0.009 mM and 0.13 mM concentrations respectively. The concentrations of Na-SU ranging from 0.5 mM - 50 mM were found to effect complete (100%) inhibition of the enzyme activity. The effects of various concentrations of NADH on Diaphorase activity in the absence or presence of aspirin metabolites were shown in Figures 1, 2 and 3. The activity of the enzyme increased with increase in NADH concentration until it reached an optimal concentration of 0.08 mM, above which the enzyme activity began to decrease progressively due to substrate (NADH) inhibition. In the presence of 10 mM Na-SA, the activity of the enzyme was inhibited by about 30% at all concentrations but up to 0.08 mM NADH (Figure 1). A concentration of 10 mM Na-GA produced a constant level of inhibition, but a more typical pattern of inhibition of the enzyme activity was observed with 5 mM Na-GA (Figure

Figure 3: The effects of various concentrations of NADH (0.02-0.20 mM) on Diaphorase activity in the absence or presence of Na-SU (0.02 mM and 0.05 mM) in human erythrocytes.

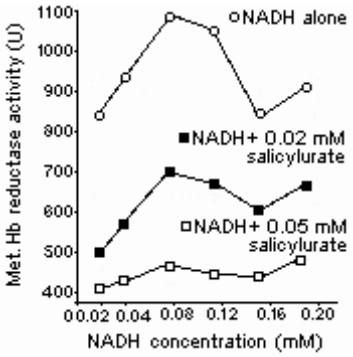


Table 3: The $Vmax^{-1}$ and Vmax values calculated from four Linewearer-Burk plots obtained from observations in duplicate on 2 hemolysate preparations.

Assay	V max^{-1} ± SD	V max* ± SD	Student's t-test
(A) NADH alone :	$0.21x10^{-1}$ ± $0.02x10^{-1}$	47.64 ± 4.56	A vs B : t=3.21 P<0.02
(B) NADH + 10mM Na-SA :	$0.41x10^{-1}$ ± $0.04x10^{-1}$	24.39 ± 2.34	
(C) NADH alone :	$0.63x10^{-1}$ ± $0.08x10^{-1}$	15.87 ± 1.23	C vs D t= 3.41 P<0.02
(D) NADH + 5 mM Na-GA :	$1.21x10^{-1}$ ± $0.09x10^{-1}$	8.26 ± 1.01	
(E) NADH alone :	$0.60x10^{-1}$ ± $0.08x10^{-1}$	16.67 ± 1.12	E vs F t=3.73 P<0.01
(F) NADH + 0.02mM Na-SU :	$1.41x10^{-1}$ ± $0.09x10^{-1}$	7.09 ± 1.05	

* : Each value is the mean ± SD of observations made in duplicate with 2 hemolysate preparations.

Figure 4: The effects of Na-SA (10 mM) on the kinetics (Vmax, Lineweaver-Burk plot) of the enzyme 'Diaphorase' in human erythrocytes.

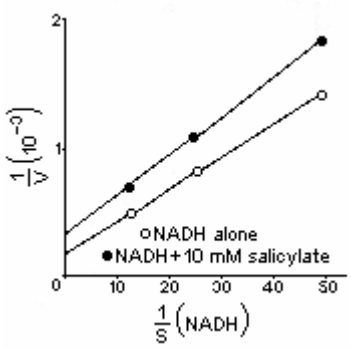
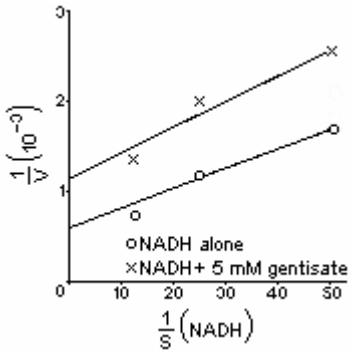


Table 4: The direct effects of aspirin metabolites on NADH-diaphorase activity in hemolysates in the presence of 0.015% dye 'DCI' as a replacement for MHb) and 0.028 mM NADH.

	NADH-diaphorase activity (U)	Percentage inhibition (%)
Control (0.015 % DCI + 0.028 mM NADH)	107 ± 3.2	00.0
Control+Na-SA (50 mM) :	55.0 ± 2.6	48.6 ± 1.96[a]
Control+Na-GA (10 mM) :	10.5 ± 1.3	90.2 ± 0.96[b]
Control+Na-SU(0.8 mM) :	3.5 ± 1.2	96.7 ± 0.86[b]

U=Units of enzyme activity; each value is the mean ± SD of 4 observations made in duplicate with 2 hemolysate preparations; 'a' is significantly different from 'b' (P<0.01); Student's t-test).

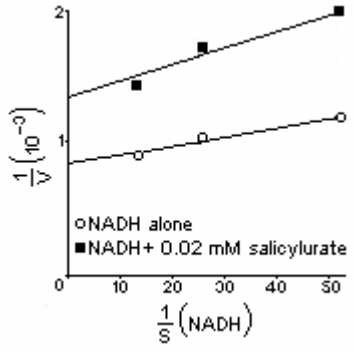
Figure 5: The effects of Na-GA (5 mM) on the kinetics (Vmax, Lineweaver-Burk plot) of the enzyme 'Diaphorase' in human erythrocytes.



2). The concentration of 0.05 mM Na-SU produced an almost constant inhibition of the enzyme, while 0.02 mM Na-SU showed more characteristic pattern of inhibition of the enzyme activity (Figure 3).

The substrate concentrations and enzyme activities (velocities parameters for Lineweaver-Burk plot, derived from the initial linear phase of the curves are stated in Table 2. The effects of Na-SA (10 mM), Na-GA (5 mM) and Na-SU (0.02 mM) on the kinetics of the enzyme are represented by mean Lineweaver-Burk plots in Figures 4, 5 and 6. respectively. The Lineweaver-Burk plots have indicated that the inhibition of the enzyme activity by all the three

Figure 6: The effects of Na-SU (0.02 mM) on the kinetics (Vmax, Lineweaver-Burk plot) of the enzyme 'Diaphorase' in human erythrocytes.



aspirin metabolites were of non-competitive in type (18). The Vmax values calculated from the Lineweaver-Burk plots and their statistical analysis are stated in Table 3.

The direct effects of aspirin metabolites on NADH-MHb reductase are shown in Table 4. Whereas 50 mM salicylate was shown to inhibit the control level by 48.6%, 10 mM Na-GA caused 90.2% inhibition and 0.8 mM Na-SU almost completely (96.7%) inhibited the enzyme activity.

The results stated in Table 5 indicates that there was no direct interaction of the aspirin metabolites with NADH.

DISCUSSION

In our previous study, we proposed that aspirin metabolites (SA, SU, GA) exert their toxic effects probably by acting at different sites of GSH and Hb metabolic pathways (8). This was based on our observation that while GA oxidized Hb to MHb, SA and SU did not show appreciable oxidative effects on Hb. But all the three metabolites (SA, SU, GA) exerted inhibition of MHb reduction significantly (8). The quantitatively major route for MHb reduction has been reported to be through NADH-MHb reductase (9, 10). The most significant finding in the present study was that Na-SU was found to be the most potent inhibitor of NADH-MHb reductase followed by Na-GA and Na-SA respectively (Table 1). This was in contrast to the effects on MHb reduction in intact red cells as reported earlier, where inhibition by Na-SU was greater than that of Na-SA but less than that of Na-GA (8). This could be possibly due to the fact that Na-SU ions do not cross through erythrocyte membrane as easily as Na-SA ions (16). In the present

Table 5: The absorbance at 366 nm of 0.036 mM NADH with or without aspirin metabolites.

Optical densities at 366 nm*							
	Aspirin metabolits alone				Aspirin metabolite + NADH		
Bank	NADH alone (0.036 mM)	Na-SA (50 mM)	Na-GA (10 mM)	Na-SU (0.8 mM)	Na-SA (50 mM) + NADH (0.036 mM)	Na-GA (10 mM) + NADH (0.036 mM)	Na-SU (0.8 mM) + NADH (0.036 mM)
0.00	0.014 ± 0.002	0.026 ± 0.004	-	-	0.041 ± 0.003	-	-
	0.014 ± 0.002	-	0.852 ± 0.005	-	-	0.865 ± 0.005	-
	0.014 ± 0.002	-	-	0.021 ± 0.002	-	-	0.036 ± 0.003

*Each value is the mean ± SD of 4 observations.

study with hemolysates, the full concentration Na-SU ions was in direct contact with the enzyme having the opportunity to express fully its inhibitory capacity.

In the studies of the effects of added NADH on the NADH-MHb reductase, it appeared that increase of NADH up to optimal concentration of 0.08 mM may reverse, to some extent, the inhibition caused by all the metabolites (Figures 1, 2 and 3). Above the optimal concentration of the substrate (NADH), substrate inhibition occurred as reported also by Worathumrong and Grimes for salicylate (16). When the datas derived from the kinetic curves (Table 2) were applied to construct the mean Linewearer-Burk plots, it was apparent that the inhibition of NADH-MHb reductase by all the three metabolites with respect to NADH as substrate was non-competitive in nature (Figures 4, 5 and 6; Table 3). This meant that the aspirin metabolites do not probably affect the 'active site' of the enzyme, but affect a region near the active site causing allosteric change of the enzyme molecules which in turn affect the catalytic activity of the enzyme (18).

However, the possibilities exist that the aspirin metabolites interact and affect the substrate 'MHb' and/or the coenzyme 'NADH' directly to cause inhibition of the NADH-MHb reductase activity. Our observation that the inhibitory effects of the aspirin metabolites could be manifested even when 'MHb' was substituted with the dye 'DCI' (Table 4), suggested that Na-SA, Na-GA and Na-SU inhibited the enzyme molecule itself rather than affecting the substrate 'MHb'. The other possibility that these metabolites might react directly with the co-enzyme 'NADH' itself, was eliminated by our observation that the absorbance of NADH was not altered and reflected the additive absorbance of metabolite and NADH when they were measured together at 366 nm (Table 5).

It was concluded, therefore, that these aspirin metabolites (Na-SA, Na-GA, Na-SU) did not react with the co-enzyme 'NADH' or the substrate 'MHb'. They exerted their effects in a non-competitive manner, perhaps by acting at a region near the active site causing allosteric (conformational) changes in the enzyme molecules and this inhibiting the catalytic activity of the enzyme 'NADH-MHb (diaphorase) reductase' in human erythrocytes.

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REFERENCES

1. Kellermeyer RW, Tarlov AR, Brewer GJ, Garson PE and Alving AS : Haemolytic effect of therapeutic drugs, clinical considerations of the premaquine-type haemolysis. JAMA 180:388-396, 1962.

2. Westring DW and Piciotta AV : Anemia, cataracts and seizures in patients with glucose-6-phosphate dehydrogenase deficiency. Arch Intern Med 118:395-392, 1966.

3. Colonna P : Aspirin and glucose-6 phosphate dehydrogenase deficiency. Br Med J 283:1189-1192, 1981.

4. Ziu MM and Giasuddin ASM : The toxicity of primaquine and effect of one of its metabolites on human erythrocytes. J Med

Res Inst 13:121-136, 1992.

5. Fraser IM and Vesell ES : Effects of primaquine and acetanilid on erythrocytes from normal and glucose-6 phosphatase dehydrogenase-deficient erythrocytes. J Pharmacol Exp Ther. 162:155-165, 1968.

6. Tarlov AR, Brewer GJ, Garcon PE and Alving AS : Primaquine sensitivity. Glucose-6-phosphatase dehydrogenase deficency: An inborn error of metabolism of medical and biological significance. Arch Intern Med 109:209-234, 1962.

7. Ziu MM and Giasuddin ASM : Plasma levels of aspirin metabolites in Libyan patients with rheumatoid arthritis and rheumatic fever. J Islam Acad Sci 1993 (In Press).

8. Ziu MM and Giasuddin ASM : In vitro chemotoxicity of aspirin metabolites on G6PD-normal and G6PD-deficient human erythrocytes. J Islam Acad Sci. 1993 (In Press).

9. Jaffe ER : Hereditary methaemoglobinaemia associated with abnormalities in the metabolism of erythrocytes. Am J Med 41:786-792, 1966.

10. Grimes AJ : Human red cell metabolism. London : Blackwell Scientific Publications, 1980.

11. Drawkins PD, Gould BJ, Sturman JA and Smith MJH : The mechanism of the inhibition of dehydrogenase by salicylate. J Pharm Prarmacol 19:355-364, 1967.

12. Grisollia S, Mendelson J and Diederich D : Inactivation of enzymes by therapeutic concentrations of aspirin and salicytate. Nature 223:79-85, 1969.

13. Sturman JA and Smith MJH : Effects of salicylates and resorcylate (2:6-hydroxbenzoate) on pathway of glucose metabolism in human red cells. Biochem Pharmacol 15:1857-1865, 1966.

14. Dacie JV and Lewis SM : Practical Haematology, 7th Edition; London : Churchill Livingstone, 1991.

15. Solem E : Glucose-6-phosphate dehydrogenase : an easy and sensitive quantitative assay for the defection of heterozygotes in red blood cells. Clin Chem 142:153-160, 1984.

16. Worathumrong N and Grimes AJ : Inhibition of methaemoglobin reduction by sodium salicylate. Br J Haematol 23:323-336, 1972.

17. Scott EM : The relation of diaphorase of human erythrocytes to inheritance of methaemoglobinaemia. J Clin Invest 39:1176-1185, 1960.

18. Stryer L : Biochemistry, 3rd edition; New York : W. H. Freeman and Company, p. 177-200, 1988.

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