CLINICAL PHARMACOLOGY and THERAPEUTICS

volume 33 number 4 April, 1983

Original articles

Microsomal incubation test of potentially hemolytic drugs for glucose-6-phosphate dehydrogenase deficiency

The in vitro metabolizing method was modified and its ability to correctly identify eight known hemolytic and nine known nonhemolytic drugs of glucose-6-phosphate (G6PD)–deficient erythrocytes was evaluated. The technique is based on inducing in vitro drug metabolism by incubation of red cells and drug with a reduced NADP–generating system in the presence of phenobarbital-induced mouse liver microsomes. Thus, this system provides a model for in vivo metabolic function. The hemolytic potential of tested drugs is indicated by the extent of loss of reduced glutathione of G6PD-deficient erythrocytes during 60-min incubations. Complete agreement between the test and literature for nonhemolytic compounds was observed. The test also correctly identified six of the eight known hemolytic drugs and failed to identify two known hemolytic drugs (acetanilide and sulfacetamide). The test was also applied to 14 drugs about which there is uncertainty regarding hemolytic potential. Of the latter, DL-α-methyldopa; α-naphthol; β-naphthol; 2,3, dimercaptopropanol; phenacetin; and menadione were found to react positively. We conclude that this in vitro assay system will be useful in predicting which new drugs will be hemolytic in G6PD-deficient patients.

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Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an inherited sex-linked abnormality involving the human erythrocyte.[8] Mutant genes producing G6PD deficiency occur primarily in populations derived from tropical areas of the world. In the United States it occurs particularly in people of African or Mediterranean descent. These individuals are asymptomatic except when exposed to a drug that induces hemolytic anemia. Drugs causing difficulties are aminoquinoline antimalarials such as primaquine, some of the sulfonamides and ni-

Supported in part by the Herrick Foundation, the Sage Foundation, the Ervin Foundation, and NIH Training grant #5-T32-GM-07544.

Received for publication Oct. 7, 1982.

Accepted for publication Jan. 10, 1982.

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trofurans, certain antipyretics and analgesics, sulfones, some vitamin K analogues, and certain vegetable foods such as the fava bean.[6, 36] Since the enzyme deficiency is common, occurring with a gene frequency of about 0.11 in the black population of the United States,[6] and since some of the drugs are in common clinical use, the prediction of drug-induced hemolysis in G6PD-deficient individuals is of clinical importance.

Many of the hemolytic drugs (e.g., primaquine) are harmless to G6PD-deficient red cells in vitro but cause hemolysis in vivo, an observation that indicates that metabolic conversion of the drugs to hemolytic intermediates may be necessary for hemolysis.[7, 27, 36] This has made it difficult to establish, by in vitro techniques, which new drugs might be hemolytic to G6PD-deficient patients. Simple incubation of the drug with G6PD-deficient red cells, as described by Beutler, causes depletion of reduced glutathione (GSH) in only a minority of situations in which the drug is known to be hemolytic in vivo.[3] Three remaining methods of identification of hemolytic compounds have involved drug administration to human subjects. The first involves carefully controlled studies like those carried out by Dern et al.[14] In the early years of investigation of G6PD deficiency they followed the survival of ^{51}Cr-labeled G6PD-deficient red cells during drug use. A second involves measuring increased amounts of $^{14}CO_2$ evolution and glucose utilization when erythrocytes from normal patients are incubated with glucose-1-C^{14} in serum after ingestion of hemolytic drugs.[16, 37] The third method of identification of hemolytic compounds has involved case reports of hemolytic reaction encountered during clinical use. Relying on case reports is undesirable in that they involve exposure of patients to an unknown risk before the danger is realized. Furthermore, it is often difficult to identify the specific drug causing hemolysis when many patients undergo multidrug therapy. In view of these problems, a dependable in vitro screening method is needed.

An effective in vitro system to screen potentially hemolytic drugs must approximate the in vivo situation by including a metabolic model that generates the hemolytically active derivatives. Results from experimentation on hydroxylated derivatives of dapsone[18, 23, 33] as well as studies of the peroxide-generating capability of hydroxylated derivatives of 8-aminoquinolines[10] have implicated the role of in vivo *N*-hydroxylation on GSH depletion, methemoglobin production, and lysis of G6PD-deficient erythrocytes. We recently published a method for modeling the in vivo metabolism of drugs by an in vitro technique involving the generation of hydroxylated derivatives.[27, 28] Briefly, this involves incubation of the drug under study with a mouse liver microsomal preparation (derived from mice) induced with phenobarbital. Additional components of the system are a reduced NADP (NADPH)–generating system and human G6PD-deficient red cells, with nondeficient red cells used as controls.

Results from this study indicated that increased hydroxylation activity of the mouse liver microsomes magnified the damaging effect of certain compounds.[27, 28] These studies also indicated decreased damaging effect when acetylated compounds of dapsone or thiazolsulfone, two sulfones, were used. It had been shown that approximately half of the human population handle such sulfones in a way that makes them hemolytic.[13] Our findings that acetylated sulfones are not damaging to G6PD-deficient cells explains the 25-year-old mystery of sulfone hemolytic bimodality. It is known that there is a genetic polymorphism affecting liver acetylation rates. About half of the population are fast acetylators, the other half slow. Fast acetylators would be relatively protected against hemolytic damage from sulfones, while slow acetylators would be vulnerable. Genetic variation in hydroxylation, well established in mice,[29] may also occur in man. Our study demonstrated for the first time that interaction between multiple pharmacogenetic systems could determine the phenotypic outcome. In this case, interaction between hydroxylation, acetylation, and G6PD deficiency was involved. Interaction between acetylation and G6PD deficiency has subsequently been confirmed by giving sulfamethazine to human subjects.[38]

The purpose of our study is to evaluate this in vitro microsomal system as a screening method for predicting the hemolytic potential of

Table I. *Classification of drugs, references, and solvents used to dissolve them in the incubation flasks*

Drug	*Solvent*	*Reference Nos.*
A. Drugs known to be nonhemolytic in G6PD deficiency		
Aspirin	Methanol	4
p-Aminobenzoic acid	Methanol	4, 13
Chloroquine (diphosphate salt)	PBS	4, 10, 15, 24
Quinidine HCl	Methanol	26, 39
Quinine SO_4	Methanol	26, 39
Sulfadiazine	Methanol	4
Sulfamerazine	Acetone	4
Sulfathiazole	Acetone	4
Tripelennamine	PBS	4
B. Drugs known to be hemolytic in G6PD deficiency		
Acetanilide	Methanol	13
Dapsone	Methanol	12
Naphthalene	Acetone	19, 42
Phenylhydrazine (hydrochloride)	PBS	13, 24
Primaquine (diphosphate)	PBS	14, 22 30, 39
Sulfacetamide (sodium salt)	Methanol	4, 13
Sulfanilamide	Methanol	4, 13, 39
Sulfapyridine	Acetone	13, 35, 39
C. Possibly hemolytic compounds in G6PD deficiency		
Acetaminophen	Methanol	
Aniline	PBS	10, 15, 21
2,3-Dimercaptopropanol	Added directly	32
DL-α-methyldopa	PBS	5, 17, 25
Menadione (sodium bisulfite salt)	PBS	20, 39, 41
Nalidixic acid	Chloroform	2
α-Naphthol	Methanol	11, 41
β-Naphthol	Methanol	34, 41
Phenacetin	Methanol	13
Sulfaguanidine	Methanol	
Sulfamethoxazole	Methanol	1, 9
Succinylsulfathiazole	PBS	
Sulfisomidine	Methanol	
Sulfisoxazole	Methanol	24

drugs. The test system has been evaluated with the use of a series of drugs already known to be hemolytic as well as a series known to be nonhemolytic. The extent of positive correlation between GSH depletion in our study and the hemolytic or nonhemolytic predictions in the literature was evaluated to establish the degree of reliability of this method. A third series of potentially hemolytic drugs about which there is uncertainty was also studied.

Methods

The in vitro liver microsomal preparation was used as described.[28] In brief, 5- to 7-wk old CD-1 male mice from Charles River Company were fasted overnight and administered a drinking solution of sodium phenobarbital and sucrose for 5 days. This procedure caused induction of the drug metabolizing systems in the microsomes. Livers were then homogenized from pooled fresh livers with cold 0.1M phosphate buffered saline (PBS), pH 7.4. The induction of liver microsomal hydroxylation activity of the prepared homogenate was estimated by Magon et al.'s[28] modification of Zannoni's[40] spectrophotometric method for measuring *p*-nitroanisole-*O*-demethylase activity.

The human G6PD-deficient (A-) red cells were freshly drawn, washed three times with cold normal saline, washed once with cold PBS,

Table II. *Percent residual GSH and standardized drug effect of test drugs in experiments with and without induced microsomes*

Drug tested	*With microsomes*			*Without microsomes*		
	No. of trials	*% Residual GSH*	*Test results*	*No. of trials*	*% Residual GSH*	*Test results*
*A. Drugs known not to be hemolytic**						
Aspirin	4	73 ± 18	90 ± 11			
p-Aminobenzoic acid	4	77 ± 16	96 ± 6			
Chloraquine (diphosphate salt)	2	71 ± 12	87 ± 6			
Quinidine HCl	2	86 ± 23	99 ± 21			
Quinine SO_4	2	56 ± 21	98 ± 9			
Sulfadiazine	3	75 ± 7	100 ± 11			
Sulfamerazine	3	60 ± 19	98 ± 10			
Sulfathiazole	2	55 ± 22	94 ± 11			
Tripelennamine	4	71 ± 8	90 ± 11			
*B. Drugs known to be hemolytic**						
Dapsone	4	32 ± 9	44 ± 13	1	127	126
Naphthalene	2	20 ± 1	26 ± 1	1	96	95
Phenylhydrazine (hydrochloride)	5	23 ± 4	35 ± 8	1	27	28
Primaquine (diphosphate salt)	2	57 ± 1	56 ± 1	3	93 ± 3	91
Sulfanilamide	3	26 ± 9	42 ± 6	1	98	97
Sulfapyridine	4	32 ± 14	45 ± 19	2	114 ± 18	111 ± 20
Acetanilide	5	73 ± 8	93 ± 5	1	108	106
Sulfacetamide (sodium salt)	4	66 ± 15	103 ± 3	2	99 ± 2	96 ± 0
C. Potential hemolytic drugs						
α-Naphthol	1	15	19	1	70	49
β-Naphthol	3	14 ± 3	24 ± 11	1	104	102
Menadione (sodium bisulfite salt)	2	28 ± 1	50 ± 11	1	51	52
Acetaminophen	4	64 ± 13	79 ± 5			
Aniline	4	58 ± 7	72 ± 5			
2,3-Dimercaptopropanol	2	15 ± 7	19 ± 7			
DL-α-methyldopa	2	50 ± 7	60 ± 8			
Nalidixic acid	2	78 ± 7	91 ± 4			
Phenacetin	3	38 ± 12	53 ± 12			
Sulfaguanidine	3	82 ± 14	101 ± 6			
Sulfamethoxazole	2	53 ± 5	95 ± 18			
Succinylsulfathiazole	2	55 ± 26	95 ± 18			
Sulfisomidine	3	73 ± 6	92 ± 6			
Sulfisoxazole	3	56 ± 23	86 ± 16			
D. Control (without drug)						
	13	73 ± 13		3	102 ± 3	

*For the most part, "known to be hemolytic" or "nonhemolytic" refers to the hemolytic potential in G6PD deficiency of the A type (black population).

and then resuspended to their original volume in PBS.

The basic in vitro mouse microsomal incubation system has been published.[28] In brief, it consisted of a separate mixture for each test drive, which combined the suspension of the freshly prepared mouse liver microsome pellet, G6PD-deficient red cells, an NADPH generating system (G6P, $MgCl_2$, $NADP^+$, G6PD dehydrogenase), and 1-mM final concentration of test drug when present.

The published method was modified for the purposes of this test as follows. The 3.7-ml incubations were scaled proportionately to 2.5 ml. Instead of using normal erythrocytes or adding the hydroxylation inhibitor SKF-525A, the controls in this study included elimination of drug, or $NADP^+$, or both from the incubation mixture. The drugs tested, the solvent in which they were dissolved just before incubation, and the references to their hemolytic or nonhemolytic nature are shown in Table I. All of the

solvents except PBS were allowed to evaporate in the flask to leave a final test compound concentration of 1 mM. (Note that 2,3-dimercaptopropanol was added directly.)

GSH levels in each flask were measured by the spectrophotometric method of Prins and Loos[31] at both the beginning and end of the 60 min incubation period at 37°. The GSH remaining after 60 min incubation was expressed as percent of initial GSH, with the exception of three drugs (2,3-dimercaptopropanol, menadione [sodium bisulfite salt], and phenylhydrazine [HCl salt]). A rapid initial loss of GSH occurred with phenylhydrazine, making it impossible to determine the initial levels accurately. Similar difficulties were found with 2,3-dimercaptopropanol and menadione with which there were extremely high initial readings, indicating some interference of the parent drug. Yet, a marked depletion of GSH was evident upon incubation. Thus, for these three drugs, the average initial GSH levels obtained from simultaneous incubations were used as estimates of basal GSH levels. To compare results between trials with different preparations, we have defined the test result as the ratio of percent residual GSH in the presence of the test agent divided by percent residual GSH in the absence of the test agent.

Results

The known nonhemolytic, known hemolytic, and possibly hemolytic compounds, along with references, are listed in Table I. We have used the drugs of known in vivo hemolytic potential (Table II, A and B) to evaluate our in vitro test system. The nine nonhemolytic drugs of Table II, A had mean test values between 87 and 100. Of these drugs, chloroquine (diphosphate salt) was found to have the lowest test result value (87, about 2 SDs above 70); this is the value we now tentatively define as the lower limit for nonhemolytic drugs. The results in Table II, B, including eight drugs known to be hemolytic in vivo, fell into two ranges; the first six listed have mean values between 26 and 56. The value of 70 as the upper limit for identifying hemolytic compounds serves to identify these six as hemolytic. The other two hemolytic drugs, acetanilide and sulfacetamide (sodium salt), had values of 93 and 103 which are in the range of the nonhemolytic drugs of Table II, A. Thus, the value of 70, while empirically chosen, appears to be a good dividing point between normal and abnormal test results.

If microsomes are not included, incubation of known hemolytic drugs with deficient red cells identified only one of these drugs (phenylhydrazine; Table II, B). When the experimentally determined value of 70 is used to evaluate the potentially hemolytic drugs of Table II, C, we find that six of the 14 drugs had positive values indicative of hemolytic potential: 2,3-dimercaptopropanol, DL-α-methyldopa, α-naphthol, β-naphthol, phenacetin, and menadione.

When normal, rather than G6PD-deficient, red cells were used in the test system along with microsomes, only phenylhydrazine gave a positive test result (data not shown).

Discussion

The principle of this new method for evaluating the hemolytic potential of drugs is based on the hypothesis that most drugs are metabolized by the liver microsomal system and become activated to hemolytic intermediates. Indeed, only one of the eight known hemolytic drugs caused GSH depletion in the absence of the microsomes (Table II, B): In the presence of microsomes, the method proved fairly reliable in separating hemolytic from nonhemolytic drugs. A natural division appeared to occur at a test result of 70. All of the known normal drugs fell above this level. The two hemolytic drugs that fell above this level were well into the midrange of the nonhemolytic drugs. Thus, we suspect that the metabolism of these two drugs is qualitatively different. It is possible that the metabolism of these drugs may not involve components available in the mouse microsomal system as it exists in our procedure. Test results for drugs of uncertain status (Table II, C) revealed that six of them (2,3-dimercaptopropanol, phenacetin, menadione, α-naphthol, β-naphthol, and DL-α-methyldopa) gave positive test results while the other eight gave negative results. Our test would predict that these drugs would be hemolytic in G6PD-deficient subjects.

Based upon our results, we have identified a test result of 70 as the critical point between hemolytic and nonhemolytic compounds. It appears so far that nonhemolytic drugs do not tend to give false-positive reactions, but that occa-

sionally a hemolytic compound will give a false-negative result. In spite of these occasional results, we conclude that this test offers considerable advantage over other in vitro and in vivo methods for the identification of potentially hemolytic drugs, with a 75% success rate in correctly identifying hemolytic drugs in contrast to the 12.5% (one of eight) success rate with incubation of the same test compounds and red cells without the induced microsomes.

We recognize that the use of this test has certain theoretical limitations. First, it depends on the liver microsomes being the site at which the tested drugs are metabolized to their active metabolites, rather than other locations within the liver or body. Second, success depends on induced mouse liver microsomes serving as an adequate model for the human microsomal system. In spite of these considerations, the method seems to be relatively accurate, and this in vitro system affords the opportunity to screen many drugs simultaneously while avoiding the risk incurred by screening procedures that expose humans to the tested drugs.[13, 16, 37]

The 17 drugs that we used for standardization include most of the drugs on which extensive human studies have been performed. It remains to be seen whether these test results coincide with positive and negative results from actual clinical experience with new drugs.

We thank Ms. Louisa La Farge and Ms. Marla Wohlman for their assistance.

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