International Journal of Pharmaceutics, 4 (1979) 75–82

STABILITY OF NITROGLYCERIN IN HUMAN AND RAT PLASMA

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(Received April 2nd, 1979)
(Accepted August 6th, 1979)

SUMMARY

The effects of concentration, temperature and silver nitrate addition on nitroglycerin stability in human plasma were examined. Degradation of nitroglycerin in rat plasma was also studied as functions of temperature and red blood cell hemolysis. Depending upon the temperature, nitroglycerin is 10–50 times more stable in human plasma than in rat plasma. Significant drug degradation, i.e. 10% loss in 40 days, still occurs when human plasma samples containing nitroglycerin are stored at −20°C. Nitroglycerin degradation in plasma apparently follows first-order kinetics and the Arrhenius relationship, and is independent of concentration up to about 150 ng/ml. Addition of silver nitrate to human plasma produced a significant increase in nitroglycerin stability. Hemolysis in rat plasma did not drastically increase nitroglycerin degradation.

INTRODUCTION

Therapeutic use of nitroglycerin has increased in recent years because of mounting clinical evidence of its beneficial effects in patients with angina (Windsor and Burger, 1975), acute myocardial infarction (Flaherty et al., 1975), and congestive heart failure (Francosia et al., 1978). The development of sensitive analytical techniques (Yap et al., 1978) for the quantitation of plasma nitroglycerin concentrations after therapeutic doses has allowed preliminary pharmacokinetic studies to be conducted in animals (Yap and Fung, 1978; Horhota and Fung, 1978, in press) and in humans (Blumenthal et al., 1977) as a function of route of administration. In such studies, it is often impossible to process the samples for analysis immediately after blood collection. Nitroglycerin degradation in rat serum is rapid, with a reported half-life of about 20 min at 37°C (DiCarlo and Melgar, 1970). The stability of nitroglycerin in human plasma has not been examined carefully. In view of the likelihood of substantial degradation of nitroglycerin in stored plasma, it is

essential that plasma nitroglycerin samples be properly preserved to minimize drug degradation prior to analysis. The proper storage conditions for plasma samples containing nitroglycerin have not been defined.

The present study examines the stability of nitroglycerin in human plasma and its dependency on concentration, temperature and silver nitrate addition. The effects of temperature and hemolysis on nitroglycerin degradation in rat plasma were also studied.

EXPERIMENTAL

Stability of nitroglycerin in human plasma

An aqueous stock solution of nitroglycerin, containing about 1 mg of nitroglycerin per ml, was prepared using a 10% lactose-adsorbate powder [1] and standardized according to the official procedure (USP, 19th revision). When the effect of concentration on nitroglycerin stability was examined, appropriate aliquots of this stock solution were added to recovered human plasma [2] to the desired concentrations, but the ratio of water to recovered plasma was maintained constant at 1.2–7.8 ml. These samples, with initial concentrations of nitroglycerin at 141, 25, and 7 ng/ml respectively, were equilibrated at 24 ± 1°C in a water bath. At selected time intervals, aliquots were withdrawn and assayed for nitroglycerin concentration, at least in duplicate, using the GLC procedure previously described (Yap et al., 1978).

The effect of temperature on nitroglycerin stability in human plasma was examined at 37, 24, 4 and −20°C and at an initial nitroglycerin concentration of approximately 25 ng/ml. The temperatures were controlled within ±2°C of those specified. At the higher temperatures, viz: 37 and 24°C, nitroglycerin stability was monitored for at least two half-lives. Because of the relative slowness of the reaction at the lower temperatures, nitroglycerin degradation was followed for slightly longer than one half-life (one week) at 4°C and shorter than one half-life (90 days) at −20°C, respectively.

Silver nitrate has been shown to inhibit nitroglycerin degradation in rat plasma (Yap et al., 1978) and serum (DiCarlo and Melgar, 1970). The effect of silver nitrate on nitroglycerin stability in human plasma was examined in this study. Spiked human plasma containing an initial nitroglycerin concentration of 25 ng/ml was separated into 200 µl-aliquots to each of which was then added 10 µl of 1 N silver nitrate. These samples, prepared in an ice-bath, were then mixed individually and incubated at 24°C. At selected intervals, aliquot samples were removed for assay. A control stability study, i.e. without silver nitrate, was carried out on the same day under identical conditions.

Stability of nitroglycerin in rat plasma

Rat blood was collected via intracardial puncture from different animals using heparinized syringes. Freshly separated plasma was used for stability studies. The effect

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[1] Nitroglycerin 10% (w/w) in lactose, lot B17-H-1, ICI America, Atlas Chemical Division, Wilmington, Dela. 19899, U.S.A.

[2] Buffalo Regional Red Cross Blood Center, from whole blood collected in 63 ml Anticoagulant Citrate Phosphate Dextrose Solution, USP. Obtained as frozen plasma and used for stability study within two months of collection.

of temperature on nitroglycerin stability in rat plasma was studied using a procedure similar to that described for human plasma, except that the time periods monitored were considerably shorter due to faster drug degradation.

In one preliminary experiment, the effect of hemolysis on nitroglycerin stability in rat plasma was explored. Blood collected from a rat was divided into two portions: one carefully treated to avoid any noticeable hemolysis (straw color plasma) and the other forcibly ejected through a narrow gauge needle to effect noticeable hemolysis (red color plasma). Nitroglycerin was added to these two plasma samples and its stability was determined at 37°C.

RESULTS

Fig. 1 shows the stability of nitroglycerin in human plasma at 24°C over a concentration range of about two orders of magnitude. It appears that nitroglycerin degradation in human plasma follows apparent first-order kinetics from about 150 ng/ml to about 2 ng/ml, and that the rate of degradation is independent of concentration over the range studied. The apparent first-order rate constant for nitroglycerin degradation, k, as determined by linear regression, was 0.062 ± 0.003, 0.057 ± 0.002 and $0.053 \pm 0.003\ h^{-1}$ (mean ± S.D. of the regressed slope) for plasma samples spiked with initial nitroglycerin concentrations of 141, 25 and 7 ng/ml, respectively. The small differences in the observed rate constants were not different as reflected by the overlap of the 95% confidence intervals of all three values. These data demonstrated that significant nitroglycerin degradation does occur in human plasma at ambient temperatures ($t_{1/2} \simeq 12$ h), and that non-linear degradation is absent over the concentration range studied.

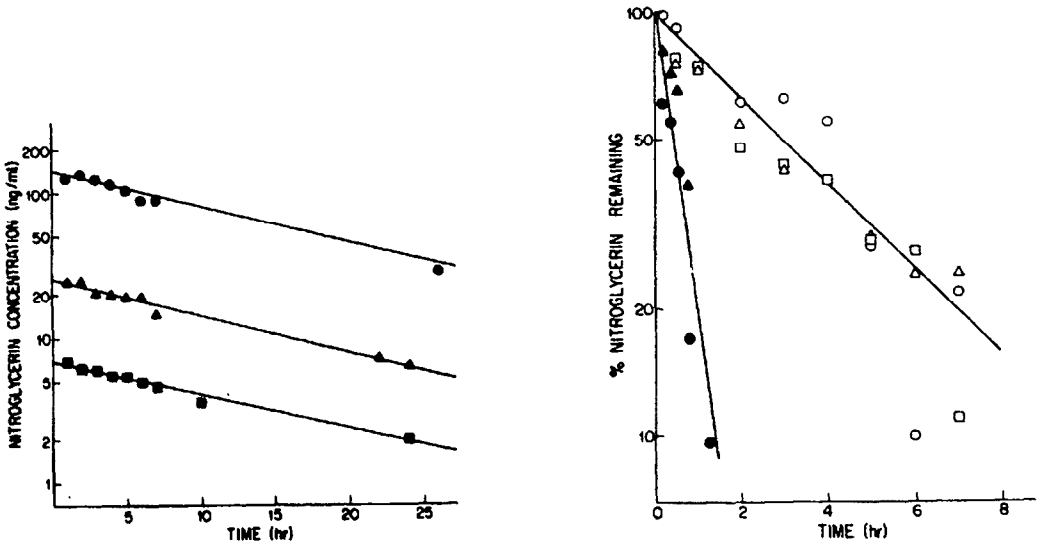

Fig. 1. Stability of nitroglycerin at different initial drug concentrations in human plasma. ●, 141 ng/ml; ▲, 25 ng/ml and ■, 7 ng/ml. Temperature = 24°C.

Fig. 2. Stability of nitroglycerin in human and rat plasma at 37°C. Open symbols, human plasma; and closed symbols, rat plasma. Different symbols represent replicate studies.

TABLE 1

EFFECT OF TEMPERATURE ON THE STABILITY OF NITROGLYCERIN IN HUMANS AND RAT PLASMA

	Human plasma		Rat plasma	
	k^a (h^{-1})	$t_{90\%}$ [b] (h)	k^a (h^{-1})	$t_{90\%}$ [b] (h)
37°C	0.238 ± 0.013	0.44	1.87 ± 0.62	0.06
24°C	0.057 ± 0.003	1.8	0.56 ± 0.28	0.19
4°C	0.0063 ± 0.0004	16.7	0.13 ± 0.06	0.81
−20°C	0.00011 ± 0.00004	958	0.0044 ± 0.001	24.0
Apparent E_a (kcal/mol) [a]	24.1 ± 1.1		19.0 ± 1.4	
ln A [a]	32.3 ± 1.7		27.4 ± 2.2	

[a] Mean ± S.D.
[b] Calculated from mean k values.

Fig. 2 compares the stability of nitroglycerin in human and rat plasma at 37°C. It is clear that plasma nitroglycerin degradation is much faster (about 9 times) in the rat than in humans. This finding is consistent with literature reports (Lee, 1973) which demonstrated marked species dependency in liver and blood nitroglycerin metabolism. The apparent first-order rate constants, as determined by linear regression, for nitroglycerin degradation in human and rat plasma at the various temperatures are presented in

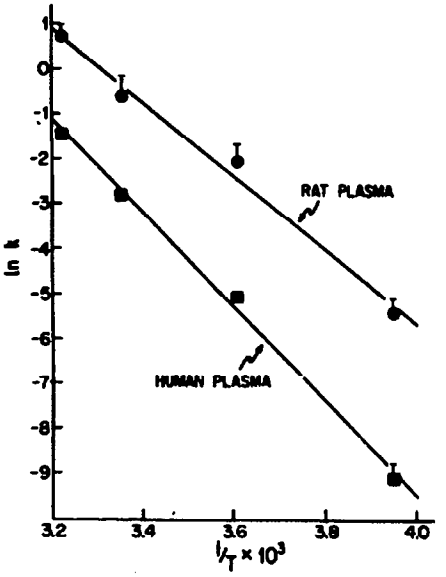

Fig. 3. Arrhenius plots for nitroglycerin degradation in human (■) and rat (●) plasma. Bars represent standard deviations. Where bars are not shown, the S.D. values are smaller than the dimensions of the symbols.

Table 1. In all cases, adherence to first-order kinetics was good: the correlation coefficients for the semilogarithmic concentration vs time plots were better than 0.95 for all studies at and above 4°C whereas those obtained for the −20°C studies were 0.85 or better. It is worthwhile to note that at −20°C (freezer temperature), significant nitroglycerin degradation still occurred in human plasma. The estimated time for 10% degradation, i.e. $t_{90\%}$, at this temperature was about 960 h or approximately 40 days. The instability of nitroglycerin was even more exaggerated in rat plasma: at −20°C the estimated $t_{90\%}$ was only 24 h.

Fig. 3 presents Arrhenius plots for nitroglycerin degradation in human and rat plasma. The Arrhenius relationship appeared to be well obeyed in both cases. The usual parameters were calculated and presented in Table 1.

In human plasma, addition of silver nitrate to a final concentration of 0.05 N produced substantial stabilization. Thus, when silver nitrate was present at 24°C, the estimated k was $0.0033 \pm 0.0014\ h^{-1}$ (mean ± S.D.) compared to a control estimated k of $0.057 \pm 0.003\ h^{-1}$ (mean ± S.D.). Addition of silver nitrate therefore enabled plasma samples containing nitroglycerin to be kept at 24°C for about 32 h with only 10% degradation.

In rat plasma, apparent hemolysis in the blood did not appear to affect nitroglycerin stability in plasma to any appreciable extent. When hemolysis was effected as described previously, the estimated k was $1.1 \pm 0.3\ h^{-1}$ (mean ± S.D.) at 37°C, compared to an estimated k in the apparently 'unhemolyzed' control of $1.7 \pm 0.2\ h^{-1}$ (mean ± S.D.). The estimated rate constants for both groups were not different since the 95% confidence intervals overlapped.

DISCUSSION

DiCarlo and Melgar (1970) were the first ones to show significant clearance of nitroglycerin in rat serum. They suggested that de-esterification of nitroglycerin by serum is enzymatic and involves the reduction of organic nitrate to organic nitrite followed by the hydrolysis of nitrite ester to inorganic nitrite. Enzymatic involvement in plasma nitroglycerin degradation is supported by numerous studies which showed that aqueous hydrolysis of nitroglycerin at neutral pH is exceedingly slow (Crew and DiCarlo, 1968; Amschler, 1974; McNiff et al., 1979) and that significant specific-acid and specific-base catalyzed hydrolysis only occurred at extreme pHs (Crew and DiCarlo, 1968). Furthermore, nitroglycerin degradation in rat serum was decreased in the presence of enzyme inhibitors such as iodoacetamide, silver nitrate and p-chloromercuribenzoate (DiCarlo and Melgar, 1970). The present study confirmed that nitroglycerin loss in rat plasma is very rapid. Even at freezer temperatures (approximately −20°C), nitroglycerin in rat plasma cannot be stored longer than 24 h without incurring more than 10% drug loss. Although nitroglycerin is more stable in human plasma, substantial drug degradation still occurred at −20°C.

A value of 0.23 mM has been reported for the Michaelis constant K_m for nitroglycerin degradation in rat serum at 37°C (DiCarlo and Melgar, 1970). The value, equivalent to 52 μg of nitroglycerin/ml, represents the substrate concentration at which the rate of reaction is half of the maximal velocity. In human plasma, first-order kinetics was

observed up to a concentration of about 140 ng/ml. It is therefore likely that the K_m value for human plasma is greater than 140 ng/ml.

Lee (1973) studied the production of glyceryl dinitrates upon incubation of nitroglycerin in human whole blood, red cells and plasma. This author showed that, after incubation at 37°C for 30 min, human whole blood and red cells produced about the same amounts of dinitrates (about 0.1 μmol/ml/min) but no measurable metabolites could be found with plasma incubation. Because the initial nitroglycerin concentration and assay sensitivity in these incubations systems were not reported, it is difficult to compare these data with the present results. Our data indicate that at 37°C, 30 min of incubation should produce about 10% nitroglycerin loss in plasma.

The excellent linearity observed for the Arrhenius relationships was somewhat unanticipated considering the enzymatic nature of plasma nitroglycerin degradation. In the simplest kinetic model involving an enzyme, E, and a single substrate, S, the observed reaction rate constant may be a function of three specific rate constants characterizing the association (k_1), dissociation (k_{-1}) and degradation (k_2) of the enzyme–substrate complex (Eqn. 1).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + \text{products} \qquad (1)$$

Thus, although each individual constant may obey the Arrhenius equation, the observed rate constant does not necessarily behave in the same manner unless (i) the enzyme protein is not denatured by extreme temperatures, and (ii) one of the reaction steps is predominantly rate-determining, or the energies of activation for all the reaction steps are of similar magnitude. In the present case, although the substrate concentrations tested are well below that of the enzyme, mechanistic interpretation of the reaction is complicated by the fact that at least two reactions and/or two enzymes (conversion of nitrate to nitrite followed by hydrolysis) may be involved in the degradation of the drug. The reasons for the apparent Arrhenius behavior of plasma nitroglycerin degradation are presently unknown.

The magnitude of the calculated apparent energies of activation (Table 1) is of some interest. Bray and White (1976) stated that in enzymatic reactions, the apparent energy of activation is dependent on substrate concentration because the reaction rate itself is dependent on the relative concentrations of the enzyme and its substrate. Thus, Schwartz (1943–44) showed that, for the hydrolysis of triglycerides by pancreatic lipase, E_a has a value of about 22 kcal/mol at low substrate concentrations, in contrast to a levelling value of about 9 kcal/mol at high substrate concentrations. In this case, nitroglycerin-degrading enzyme systems in both human and rat plasma also gave E_a values close to 20 kcal/mol, at substrate concentrations well below enzyme saturation.

Addition of silver nitrate produced an approximate 17-fold increase in nitroglycerin stability in human plasma at 24°C. Significant protein precipitation occurred when silver nitrate was added to plasma. Heavy metal ions such as silver are known to bind to SH groups on proteins (Lehninger, 1975). Thus the mechanism of inhibition probably arises from precipitation and/or destruction of plasma enzyme proteins which are responsible for nitroglycerin degradation.

The use of blood bank plasma may give rise to concerns that the degradative process is

potentially affected by the method of collection (i.e. addition of the anticoagulant citrate phosphate dextrose solution) and storage conditions. In order to address this question, a stability study was also conducted in pooled plasma freshly collected from four volunteers in heparinized tubes. The observed rate constants, obtained from linear regression of data from single runs at 4, 24 and 37°C, were 0.010 ± 0.0005, 0.057 ± 0.006 and 0.193 ± 0.017, respectively (slope ± S.D.). These values were essentially identical to those obtained with the blood bank plasma. A less detailed study also indicated that nitroglycerin stability at −20°C, was not altered in fresh plasma. Based upon these results it appears that nitroglycerin degradation is similar in both fresh and blood bank plasma.

The present study demonstrates that nitroglycerin is unstable in human plasma, and even more so in rat plasma. Human plasma containing nitroglycerin can be preserved at −20°C for only about 40 days with 10% degradation. Rat plasma containing nitroglycerin cannot be preserved longer than one day at −20°C without more than 10% drug loss. It should be noted that the stability studies described involved slightly diluted plasma, i.e. about 15% water in human plasma and 5% water in the rat plasma. Degradation of nitroglycerin in undiluted plasma could possibly be somewhat faster. Because intersubject variation in systemic nitroglycerin clearance is quite large (Yap and Fung, 1978; Oh and Reid, 1979), nitroglycerin stability in individual plasma samples may also vary considerably.

The prudent approach therefore would seem to employ a combination of low temperature storage and silver nitrate inhibition for preservation of nitroglycerin in plasma. Even under these conditions, it would appear unwise to leave plasma samples containing nitroglycerin unprocessed over a 1–2 month period.

ACKNOWLEDGEMENT

Supported in part by NIH Grants HL 22273 and GM 20852.

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