International Journal of Pharmaceutics, 8 (1981) 323–329
Elsevier/North-Holland Biomedical Press

ABSENCE OF METABOLITE IN THE DISAPPEARANCE OF NITROGLYCERIN FOLLOWING INCUBATION WITH RED BLOOD CELLS

CHIEN-CHIN WU, THEODORE SOKOLOSKI, MARVIN F. BLANFORD and ALLAN M. BURKMAN

College of Pharmacy, Ohio State University, 500 W. 12th Avenue, Columbus, Ohio 43210 (U.S.A.)

(Received March 31st, 1981)
(Accepted April 13th, 1981)

SUMMARY

When nitroglycerin was incubated at 37°C with human red blood cells obtained from two subjects and from outdated blood, the rate of loss of nitroglycerin observed was similar to that reported by others. At an initial concentration of 180 ng/ml nitroglycerin the loss followed a first-order process with a half-life of about 10–15 min depending on the source of the cells. Using an assay that could clearly follow the production of the major metabolites, 1,2- and 1,3-dinitroglycerin, it was found that none were present in the medium over the entire course of nitroglycerin loss. This finding shows that the the assumption by other workers, that the loss of nitroglycerin in blood is an enzymatic reaction, is not valid.

INTRODUCTION

The rapid disappearance of nitroglycerin following incubation with human blood (Armstrong, et al., 1980; Lee, 1973) has been assumed to occur via an enzymatic mechanism. It has also been suggested that the slower loss of nitroglycerin in human and rat plasma (Maier et al., 1979) and in rat serum (Di Carlo and Melgar, 1970) also could be enzymatic. Armstrong et al. and Maier et al. studied the disappearance of nitroglycerin by GC analysis without following metabolite formation. Di Carlo and Melgar, and Lee used a thin-layer chromatographic separation of labelled compounds and did follow metabolite formation. Studies reported in the present communication show that the loss of nitroglycerin in human blood is rapid but, by using an assay able to detect metabolites, it is seen that the reaction is not enzymatic in nature.

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MATERIALS AND METHODS

Nitroglycerin stock solutions were prepared from an alcoholic extract of a 10% lactose adsorbate [1]. The metabolites. 1,2- and 1,3-dinitroglycerin were prepared from 1,2-dibromopropanol [2] and 1,3-dibromopropanol [3] using the method of Dunstan et al. (1965). The purity of the compounds was determined by thin-layer chromatography and HPLC and the solutions standardized as reported earlier (Yuen et al., 1979).

An electron capture gas chromatograph [4] was used in conjunction with a data processor [5]. 9% QF-1-0065 [6] was used as the stationary phase on 60/80 mesh Supelcoport [7]. The column was silanized glass, 2 mm × 50 cm. The temperature of the injection port, the column, and the detector was 150, 135 and 200°C, respectively. The carrier gas, 95% argon/5% methane, was used at a flow rate of 16 ml/min. The electrometer range was 1×10^{-11}. The short and highly loaded column was used to minimize thermal decomposition and adsorption of nitroglycerin and its metabolites. The detector showed linear response to nitroglycerin and 1,2- and 1,3-dinitroglycerin over the concentration range used. Under these operating conditions the retention times of the dinitroglycerins, nitroglycerin, and the internal standard, 1-fluoro-2,4-dinitrobenzene [8], were 1.1, 2.2 and 2.7 min, respectively (Fig. 1C). The system used will not seperate the two dinitroglycerins but it optimizes their combined detection. The ratio of detector responses, nitroglycerin : dinitroglycerin, is 2:1. The concentration of nitroglycerin (or dinitroglycerin) in the various samples was determined either relative to the internal standard or via an external standard where comparisons were made relative to known amounts of nitroglycerin added to blanks. The assay precision is ±8%.

Data using resuspended cells are reported in this paper since the loss of nitroglycerin is the same in these systems as in whole blood (Armstrong et al., 1980; our own work) and since the resuspended cell represents a more clearly defined system. The cells were obtained from fresh heparinized blood donated by two different subjects and from outdated blood obtained from the OSU Hospital Blood Bank. The blood was centrifuged at 1200 rpm at 5°C for 20 min. The plasma was replaced with normal saline and the cells recentrifuged. The procedure was repeated 3 times. With outdated blood, the initial number of cells per unit volume was counted and the final volume adjusted so that the number of cells per unit volume was approximately the same as that of the fresh blood used.

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[1] Nitroglycerin 10% (w/w) in lactose, Lot K17-0-H, ICI America, Atlas Chemical Division, Wilmington, Dela. 19899, U.S.A.
[2] Eastman Kodak, Lot A5A, Rochester, N.Y. 14650, U.S.A.
[3] Eastman Kodak, Lot B6C, Rochester, N.Y. 14650, U.S.A.
[4] 3700 Series dual column gas chromatograph, Varian Instrument Division, 611 Hansen Way, Palo Alto, Calif. 94303, U.S.A.
[5] Shimadzu Recording Data Processor. Chromatopac C-RIA Shimadzu Scientific Instruments, Oakland Ridge Industrial Center, 9147-H Red Branch Road, Columbia, Md. 21045, U.S.A.
[6] Analabs, 80 Republic Drive, No. Haven, Conn. 06473, U.S.A.
[7] Supelco, Supelco Park, Bellefonte, Pa., 16823, U.S.A.
[8] Eastman Organic Chemicals, Distribution Products Ind. Rochester, N.Y., U.S.A.

Two ml of the resuspended cell preparations were transferred to each of a series of 12 ml silanized tubes and warmed to 37°C in a water bath for 20 min. Seventy-five μl of a nitroglycerin solution in normal saline (4.8 μg/ml) were added to each tube making the final concentration in each tube 180 ng/ml. The tubes were gently agitated using a rotator submerged in a water bath at 37°C. Periodically over an interval of 60 min, a tube was removed, 50 μl of a solution of internal standard (0.0005%) added (if necessary), and then extracted twice with 3 ml of ethylacetate [9]. Both organic extracts were combined and concentrated to 1 ml over a stream of nitrogen. Five μl of the solution were injected into the gas chromatograph for determination of nitroglycerin and metabolite content.

Since thermal decomposition of nitroglycerin may occur in a GC procedure (Camera and Pravisani, 1964), studies were undertaken to determine the extent of such decomposition in the method used. Very pure nitroglycerin was obtained by extracting an aqueous solution of the drug with pentane [9], then removing the pentane using nitrogen, redissolving the nitroglycerin in water and repeating the pentane extraction two more times. The purity of the nitroglycerin in aqueous solution was determined using HPLC. A C-18 reverse-phase column was used with 60% methanol in water as the mobile phase. The final aqueous nitroglycerin solution was extracted with ethyl acetate and the nitroglycerin concentration of the ethyl acetate solution determined by HPLC. This ethyl acetate solution was used as our standard solution. Using this solution in our GC method, it was found that a 12% conversion of nitroglycerin to dinitroglycerins occurred via thermal decomposition on the column.

To determine the extraction efficiency of 1,2- and 1,3-dinitroglycerin, various volumes of solutions of each metabolite (5 μg/ml) were added to red cell suspensions to obtain final concentrations ranging between 60 and 180 ng/ml. The solutions were extracted with ethyl acetate and the amounts extracted measured by the GC assay. The extraction efficiency found for each metabolite was 84%. The extraction efficiency for nitroglycerin was 75%.

RESULTS

In general, the ethyl acetate extraction of suspended cells containing no nitroglycerin gave uncomplicated chromatograms. Fig. 1A shows the chromatogram for the ethyl acetate extract of the resuspended cells both from subject 1 and from outdated blood. Fig. 1B, for subject 2, showed a very small potential interfering peak at the place where metabolites appear. To demonstrate the resolution of nitroglycerin from its metabolites the cell suspension of outdated blood was spiked with nitroglycerin, dinitroglycerins and internal standard. The chromatogram for a solution containing 90 ng/ml nitroglycerin and 90 ng/ml 1,2-dinitroglycerin (Fig. 1C) demonstrates that clear separation of these components is easily achieved. The 1,3-dinitro metabolite has a retention time identical to that of the 1,2-derivitive.

[9] Baker Resi-Analyzed for pesticide analysis, J.T. Baker Chem. Phillipsburg, N.J. 08865, U.S.A.

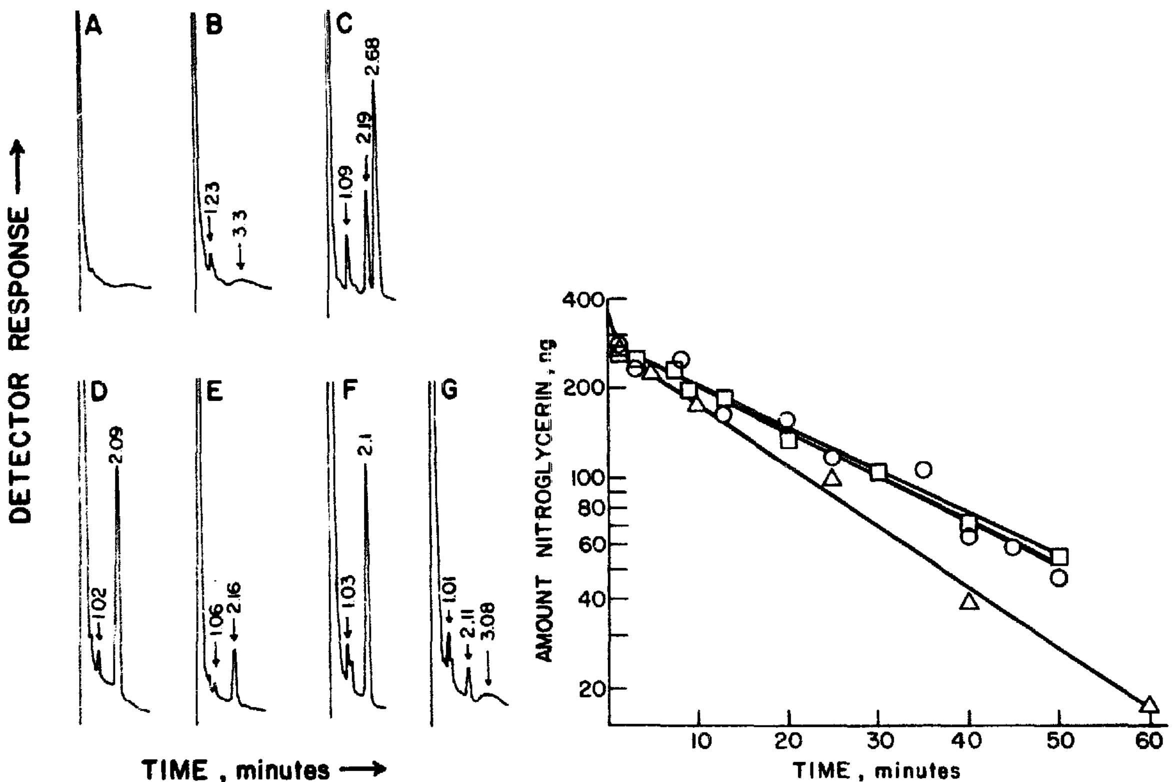

Fig. 1. Chromatograms (response vs time in min) for the ethylacetate extracts of red blood cells suspended in normal saline at 37°C. The numbers above the peaks are retention times in minutes: A, blank for both subject 1 and outdated blood; B, blank for subject 2; C, outdated blood spiked with 90 ng/ml 1,2-dinitroglycerin (1.09 min), 90 ng/ml nitroglycerin (2.19 min), and 1-fluoro-2,4-dinitrobenzene (internal standard, 2.68 min); D, outdated blood with 180 ng/ml added nitroglycerin sampled at zero time; E, outdated blood initially at 180 ng/ml nitroglycerin sampled at 40 min; F, subject 2 with 180 ng/ml added nitroglycerin sampled at zero time; G, subject 2 initially at 180 ng/ml nitroglycerin sampled at 40 min.

Fig. 2. Relationship between the logarithm of the amount of nitroglycerin remaining in 2 ml of resuspended cells as a function of time in minutes. The initial concentration of nitroglycerin was 180 ng/ml and the suspension stored at 37°C. The data are for cells from: ○, outdated blood; □, subject 1; △, subject 2.

Analysis of samples of resuspended cells incubated with 180 ng/ml of nitroglycerin resulted in chromatograms represented by Fig. 1D–G. Fig. 1D and F are zero time determinations for outdated blood and subject 2, respectively and Fig. 1E and G are for the same cell studies but sampled at 40 min. From chromatograms such as those in Fig. 1 together with standard curves for the response of known amounts of nitroglycerin in solution, it is possible to calculate the amount of nitroglycerin remaining at each of the several sampling times. When these data are plotted as the logarithm of amount remaining vs time, the result obtained is a linear relationship as presented in Fig. 2.

DISCUSSION

It is clear when using cells from outdated blood (Fig. 1D and E) that as the concentration of nitroglycerin decreases with time there is no indication that metabolite forms. The chromatograms show that there is a small amount of material that has a retention time similar to that of metabolite (1.02 and 1.06 min, respectively) in samples at 0 and 40 min post-incubation. The small amounts appearing are due to thermal decomposition of nitroglycerin on the column where about 12% decomposition is expected. Also, the peak corresponding to the metabolite decreases as nitroglycerin decreases and thus substantiates the thermal decomposition hypothesis. The very small metabolite peak in the chromatograms using the second subject's cells (Fig. 1F and G) does not change with time although the content of nitroglycerin decreases. The small peak should decrease but experimental error and the observation that a potentially interfering material is extracted by ethyl acetate from the subject's cells (Fig. 1B) may explain this observation.

Armstrong et al. (1980) found a rapid loss of nitroglycerin when the drug was incubated with human whole blood or resuspended cells at 37°C ($t_{1/2}$ = 6.2 and 6.6 min, respectively). They did not assay for metabolites but assumed the reaction was enzymatic based on the work of Marcus et al. (1978) who isolated glutathione transferase ρ from fresh and outdated blood. The enzyme is different from hepatic glutathione transferases α-ϵ – the δ form of which is very active in the metabolism of nitroglycerin. Marcus et al. state that the ρ enzyme differs from the α-ϵ forms in 'its inability to catalyze ... the denitration of nitrate esters' including nitroglycerin. Armstrong et al. presented calculations of potential nitroglycerin loss in blood based on a presumed specific enzyme activity for the glutathione transferase ρ of 1 360 ng/min/mg protein. This value is derived from a table in the Marcus paper which is not a specific activity but 'represents the lower limit of the assay at the highest enzyme concentration used'. Lee (1973) used labelled nitroglycerin and a thin-layer chromatographic separation to study the loss of nitroglycerin at high initial concentrations (1 mg/ml) in blood as well as the formation of metabolites. He reports that whole blood metabolizes nitroglycerin to dinitroglycerin at a rate of 0.096 μmol/ml/min (22 μg/ml/min). This activity would predict a rate of loss of nitroglycerin from blood that is higher than any observed rate of loss from the body especially at levels expected following sublingual dosing (2 ng/ml) or following i.v. infusion (62 ng/ml) (Armstrong et al., 1980). Armstrong in fact reports a biological half-life of nitroglycerin of 1.9 min following i.v. infusion. This rate would not be expected if Lee is correct.

Maier et al. (1979) studied the stability of nitroglycerin in human and rat plasma and suggested that the reaction was enzymatic based on the work of Di Carlo and Melgar (1970). These latter workers studied the decomposition of nitroglycerin in rat serum ($t_{1/2}$ = 20 min) and unlike Maier et al. also measured the appearance of metabolites using labeled nitroglycerin and thin-layer chromatography. Maier et al. report about a 9-fold slower loss of nitroglycerin in human plasma compared to the rat ($t_{1/2}$ = 22 min at 37°C in the rat). The loss of nitroglycerin in plasma is much slower than in blood. In human plasma the half-life is 53.4 min according to Armstrong et al. (1980) and 175 min according to Maier et al. (1979) who used a slightly diluted plasma. Because the plasma loss is slower than that in blood the question is raised if it is enzymatic since the decomposition in blood is not. Based on some of our preliminary studies concerning the nitro-

glycerin reaction with proteins, we suspect that nitroglycerin can readily react with sulfhydryl groups to form denitrated compounds, and suggest that perhaps this is what is occurring in the plasma systems. Studies in our laboratories will further explore this possibility.

Further evidence exists that the decomposition of nitroglycerin in blood is non-enzymatic. Lang et al. (1972) followed the blood levels of nitroglycerin as a function of time in control and completely eviscerated rats after i.v. injection. In the control animals there was a rapid disappearance of nitroglycerin ($t_{1/2}$ <1 min) and a simultaneous appearance of metabolites peaking in 2–5 min and disappearing with a half-life of 3–4 h. In direct contrast, the disappearance of nitroglycerin from the blood of eviscerated animals had a half-life of 7–8 min where there was no increase with time in the initial very low concentration of metabolites thus suggesting a non-enzymatic loss from blood.

The half-life for the first-order loss of nitroglycerin from resuspended cells of subject 1 and from outdated blood was 10 min and that for subject 2, 15 min (Fig. 2). All studies were conducted at an initial nitroglycerin concentration of 180 ng/ml. The half-life found by Armstrong et al. (1980) was 6.6 min at an initial concentration of 50 ng/ml. Some of our own studies using whole blood and resuspended cells from outdated blood at initial concentrations of 60 ng/ml gave rate constants similar to those of Armstrong ($t_{1/2}$ ~7 min). It is our observation that the rate constant for the loss of nitroglycerin from blood decreases as the initial concentration of nitroglycerin increases. At very high initial concentrations (100 μg/ml) the rate is essentially zero.

Although we cannot offer a mechanism for the loss of nitroglycerin in blood at the present time, we feel that the mechanism cannot be enzymatic but probably is physical in nature since nitroglycerin is lost without the formation of dinitroglycerin. Studies in progress should establish the mechanism.

The observations of Armstrong et al. and Maier et al. still retain their significance. The loss of nitroglycerin in blood and plasma is real, having a finite measureable rate. It is only the interpretation of mechanism that is lacking. An understanding of the mechanism is important for valid interpretation or prediction of blood effects on nitroglycerin disposition.

ACKNOWLEDGEMENTS

Supported in part by NIH Grant HL 23303 and the Eli Lilly Company, Indianapolis, Ind., U.S.A.

REFERENCES

Armstrong, J.A., Slaughter, S.E., Marks, G.S. and Armstrong, P.A., Rapid disappearance of nitroglycerin following incubation with human blood. Can. J. Physiol. Pharmacol., 58 (1980) 459–462.

Camera, E. and Pravisani, D., Separation and analysis of alkylpolynitrates by gas chromatography. Anal. Chem., 36 (1964) 2108–2109.

Di Carlo, F.J. and Melgar, M.D., Nitroglycerin biotransformation by rat blood serum. Biochem. Pharmacol., 19 (1970) 1371–1379.

Dunstan, I., Griffiths, J.V. and Harvey, S.A., Nitric esters. Part I. Characterisation of the isomeric glycerol dinitrates. Chem. Soc. (Lond.) (1965) 1319–1324.

Lang, S., Johnson, E.M. Jr. and Needleman, P., Metabolism of and vascular responses to glyceryl trinitrate in the eviscerated rat. Biochem. Pharmacol., 21 (1972) 422–424.

Lee, H.L., The metabolism of glycerol trinitrate by liver and blood from different species. Biochem. Pharmacol., 22 (1973) 3122–3124.

Maier, G.A., Poliszczuk, A. and Fung, H.-L., Stability of nitroglycerin in human and rat plasma. Int. J. Pharm., 4 (1979) 75–82.

Marcus, C.J., Habig, W.H. and Jakoby, W.B., Glutathione transferase from human erythrocytes. Arch. Biochem. Biophys., 188 (1978) 287–293.

Yuen, P.H., Denman, S.L., Sokoloski, T.D. and Burkman, A.M., Loss of nitroglycerin from aqueous solution into plastic intravenous delivery systems. J. Pharm. Sci., 68 (1979) 1163–1166.