International Journal of Pharmaceutics, 12 (1982) 331–340
Elsevier Biomedical Press

Formation of mono- and dinitrate metabolites of nitroglycerin following incubation with human blood

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(Received January 12th, 1982)
(Accepted May 7th, 1982)

Summary

Tritium-labeled GTN ([^{3}H]GTN) was incubated with fresh human blood, pooled plasma and buffer (pH 7.4). Levels of [^{3}H]GTN and [^{3}H]metabolites were determined by selective extractions and normal phase HPLC. After incubation with blood, the decline in [^{3}H]GTN was first-order with a half-life of 15 min (17 ng/ml) and 22–32 min (1.6 ng/ml). The GTN half-lives varied with the source of blood and the initial GTN concentration. The half-life during incubation of [^{3}H]GTN with plasma was 8-fold longer (202 ± 5 min). No detectable degradation of [^{3}H]GTN occurred during incubation with buffer for 21 h. After a two-hour incubation of [^{3}H]GTN in blood, the majority of the [^{3}H]-label (99 ± 9%) was accounted for by the presence of the mononitrate (4.7 ± 1.4% 1-GMN and 23.0 ± 4.3% 2-GMN) and the dinitrate metabolites (19.3 ± 5.7% 1,3-GDN and 52.9 ± 6.9% 1,2-GDN). Similarly, after a 21-h incubation with plasma, 100 ± 8% of the [^{3}H]-label was accounted for by the presence of: 1,3-GDN (62.5 ± 5.4%); 1,2-GDN (31.5 ± 5.4%); 1-GMN (3.1 ± 1.0%); and 2-GMN (2.9 ± 3.2%).

Introduction

Nitroglycerin (GTN) is metabolized by a non-specific glutathione-S-transferase in hepatic tissues (Habig et al., 1976). A different isozyme than that found in liver was identified in human erythrocytes (Marcus et al., 1978). These authors could not demonstrate measurable metabolism of GTN by this purified enzyme. Lee (1973)

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0378-5173/82/0000–0000/$02.75

demonstrated the conversion of GTN to 1,2- and 1,3-glyceryldinitrates (1,2-GDN and 1,3-GDN) after incubation with blood. However, he used concentrations of GTN that would not be physiologically obtainable (1 mg/ml). Also, he was not able to detect the formation of glycerylmononitrates (GMNs). Armstrong et al. (1980a) demonstrated the disappearance of physiologically relevant concentrations of GTN following incubation with blood. Even though these workers did not measure the appearance of metabolites, they assumed that the loss of GTN was due to metabolism.

More recently, Wu et al. (1981) attempted to measure the formation of metabolites (GDNs) following incubation of GTN with human erythrocytes. These investigators noted that even though levels of GTN decreased with time, the metabolites could not be detected. They concluded that the reaction of GTN with blood was not enzymatic but that the loss was physical in nature.

This study was designed to elucidate the mechanism by which GTN might be lost (i.e. either a physical or metabolic loss). After incubating [^{3}H]GTN with blood, the radioactive label may be extracted and quantitated. If irreversible drug binding occurred, it could be detected. Thus, use of the tritium label allows a mass balance. We wish to report that, on incubation of [^{3}H]GTN with fresh human blood, both dinitrate and both mononitrate (GMNs) metabolites are formed.

Materials and methods

Heparinized blood was obtained from 3 volunteers immediately before the incubation experiments. Silanized Erlenmeyer flasks containing 50 ml of blood were gently shaken in a Dubnoff [1] metabolic incubator at 37°C. After a 10-min equilibration period, an aliquot of tritiated-GTN [2] (1.36 μg/ml in normal saline) was added to the blood to make a final concentration of 17 or 136 ng/ml GTN. One incubation was performed at 17 ng/ml and 3 incubations at 136 ng/ml. Separate 50 ml aliquots of blood from one volunteer were incubated at both concentrations.

Aliquots of blood were withdrawn from the flasks at the following time periods: 0, 10, 20, 30, 40, 50, 60, 90 and 120 min. Two aliquots of blood were treated at each time interval: 1 ml of whole blood was extracted directly and 2 ml of blood from which 1 ml of plasma was obtained and was subsequently extracted. Plasma was obtained by centrifuging [3] the 2 ml aliquot of blood for 20 s at 12,800 *g*. Each plasma and blood sample was first extracted with 10 ml of pentane [4] by shaking on a

[1] Dubnoff metabolic shaking incubator, Precision Scientific, Chicago, IL.

[2] Tritiated nitroglycerin ([2-^{3}H]GTN, 150 μCi/mg; radiochemical purity = 97.8%) was kindly provided by Dr. Alec Keith of Key Pharmaceuticals, Miami, FL.

[3] Eppendorf microcentrifuge model 5412, Brinkman Instruments, Westbury, NY.

[4] All solvents were of HPLC quality, distilled in glass, Burdick and Jackson Laboratories, Muskegon, MI.

vortex mixer [5] for two minutes in a 16 × 150 mm silanized test tube with a teflon-lined cap [6]. After transferring the pentane layer to a scintillation vial [7], each sample (blood and plasma) was again extracted with 10 ml of ether. The ether was then transferred to a new scintillation vial. Ten ml of cocktail [8] was added to each vial. Tritium content was determined by scintillation spectrometry [9].

Duplicate aliquots of blood and plasma (obtained from the blood incubation) were extracted at the 120 min time interval. The duplicate ether extracts were evaporated under a stream of nitrogen at room temperature. The residues were dissolved in 100 μl ethanol containing unlabeled metabolites [10]. A 20 μl aliquot was counted for radioactivity. Another 20 μl aliquot was chromatographed using normal phase HPLC.

HPLC was performed on a liquid chromatograph equipped with a solvent delivery system [11] and manual injector [12]. Detection of GTN metabolites was carried out with a fixed-wavelength UV detector [13] at 214 nm. The prepacked silica column [14] was eluted with hexane–ethanol (96 : 4, v/v) at a flow rate of 3.0 ml/min. Under these conditions, the detection limit for the GDNs was 4 ng injected while the GMN detection limit was 15–20 ng injected (signal/noise ratio = 2). All 4 metabolites are resolved from each other and GTN.

After sample injection, fractions of the HPLC effluent were collected into scintillation vials. Those fractions containing metabolites (as indicated by the UV detector) were noted. Each fraction was counted for tritium by scintillation spectrometry as previously described.

As a control experiment, [^{3}H]GTN (136 ng/ml) was incubated both in buffer [15] (pH 7.4) and human plasma. The buffer was chosen so as to mimic both the pH and electrolyte composition of blood. Human plasma was obtained from 12 different volunteers and pooled before use. [^{3}H]GTN was incubated (37°C) in triplicate in buffer and aliquots (1 ml) were withdrawn at 0, 2 and 21 h. Triplicate incubations with plasma were also performed and aliquots (1 ml) were withdrawn at 0, 15, 30, 45, 60, 75, 90, 105, 120, 180 and 240 min, and at 21 h. Each aliquot of buffer and plasma was extracted and tritium content determined as described above.

Duplicate aliquots of plasma were extracted at the 21-h time interval. These duplicate extracts were chromatographed (HPLC) as described above to determine metabolite composition.

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[5] Vari-whirl mixer, VWR Scientific, San Francisco, CA.
[6] Corning Glass Works, Corning, NY.
[7] Research Products International, Elk Grove Village, IL.
[8] Aquasol, New England Nuclear, Boston, MA.
[9] Tri-carb I, model 3375, Packard Instruments, Downers Grove, IL.
[10] Kindly provided by Midwest Research Institute, Kansas City, MO.
[11] Model 8500 pump, Varian Instrument Group, Palo Alto, CA.
[12] Model 7125 with silica loop-column, Rheodyne, Berkeley, CA.
[13] UV III monitor, model 1203, LDC, Riviera, FL.
[14] 4.6×250 mm Partisil, 5 μm, Whatman, Clifton, NJ.

Results and discussion

Pentane and ether were chosen as extraction solvents because of their respective partitioning properties. GTN is preferentially extracted into pentane relative to the drug's more polar metabolites. In pentane, 75% of GTN is extracted but only 9% of the GDNs and less than 1% of the GMNs will be extracted under the conditions described above (i.e. single extraction, 10 : 1 organic solvent–biological fluid, 2 min contact time). In contrast, 100% of the GDNs and 60% of the GMNs will partition into an ether extraction. Thus, GTN and metabolite levels may be approximated by the tritium content in the pentane and ether extracts, respectively.

The levels of radiolabeled compounds (equivalent to GTN) in the ether and pentane extracts as a function of incubation time are shown in Fig. 1A. Concentrations of GTN and metabolites, after normalizing for GTN and metabolite extraction efficiencies, are shown in Fig. 1B. Similar profiles were obtained for each of the 4 incubations. The disappearance of GTN may be followed in both plasma and whole blood and is apparently a pseudo-first-order process. At the later time points, GTN concentrations are low with respect to metabolite concentrations. The apparent loss of linearity at these low GTN concentrations may be caused by the partitioning of GDNs into pentane (since pentane can extract approximately 9% of the GDNs). Under these conditions (i.e. very low GTN and high GDN concentrations), the pentane extract may no longer be specific for GTN. The GTN half-life was found to vary with the blood source and the initial GTN concentrations (Table 1). The initial concentrations of GTN used in this study fall within the range of arterial levels reported by Armstrong et al. (1980b) after i.v. infusion. Wu et al. (1981) and Sokoloski et al. (1981) reported a similar observation, that the GTN half-life increased with increasing initial GTN concentration. In a linear system, the half-life should be independent of concentrations. One might speculate that such behavior (increasing half-life with increasing GTN concentration) may be caused by either substrate or end-product enzyme inhibition.

Fig. 1 also shows that, as the concentration of GTN decreases, the concentration of polar, ether extractable (radiolabeled) compounds increases. These curves reflect the metabolism of GTN and the accumulation of metabolites. This accumulation can be measured in both plasma and whole blood. The half-life for the appearance of total metabolites is identical to that for the loss of GTN.

The curve at the bottom of Fig. 1A shows the relationship between the amount of GTN-related material not extracted from plasma and the time of incubation. Since only 60% of the GMNs are extracted by ether, this curve may reflect the accumulation of GMNs in plasma. The amount of radioactivity remaining in extracted whole blood was not determined.

Table 2 shows the blood–plasma ratios (B/P) of GTN and total metabolites with time. During the log-linear period of GTN metabolism, the blood–plasma ratio of GTN remained relatively constant. These ratios are similar to that reported by Fung

[15] Normosol-R pH 7.4, Abbott Laboratories, North Chicago, IL.

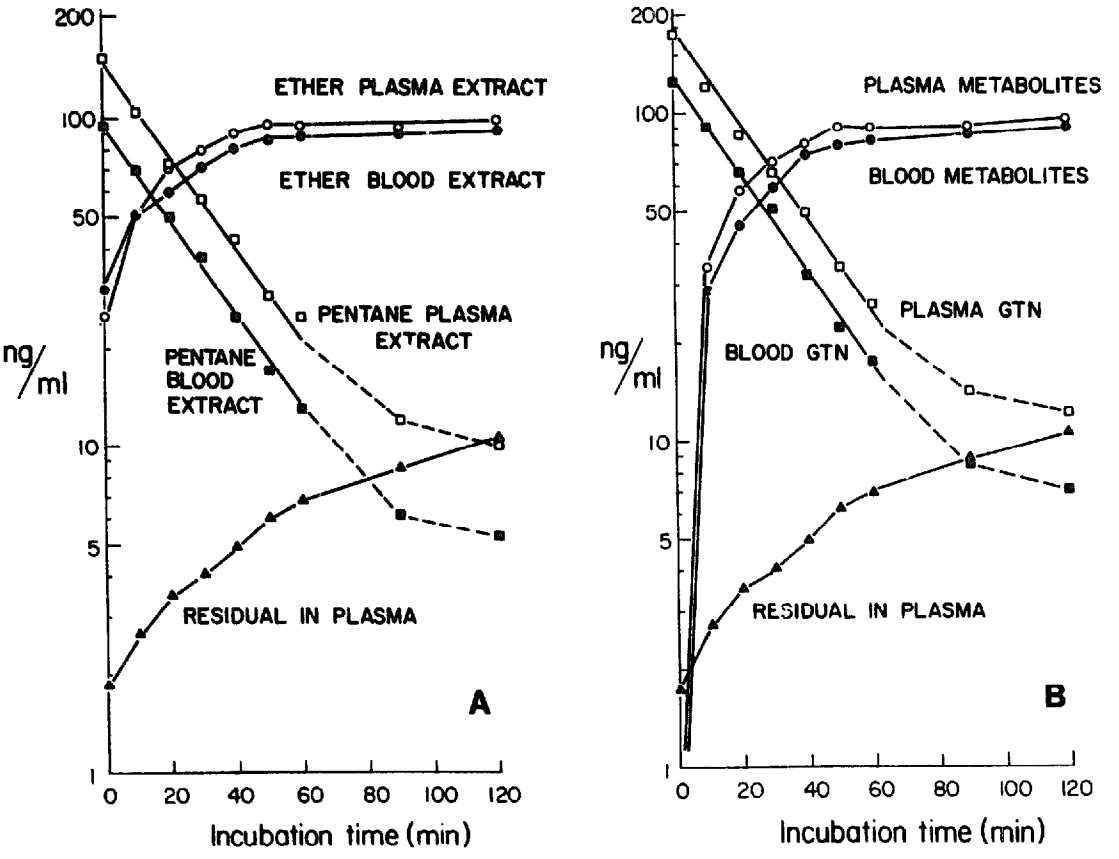

Fig. 1. In vitro metabolism of [^{3}H]GTN to [^{3}H]metabolites by human blood. Figures show the loss of [^{3}H]GTN in both plasma (□) and blood (■) and the accumulation of [^{3}H]metabolites (○, plasma; ●, blood) with time. Bottom curve shows the residual concentration of radioactivity in plasma (▲) after extractions. Fig. 1A shows the concentration of radioactive material (as GTN) found in the extracts. These data were normalized for GTN and metabolite extraction efficiencies to show concentrations of [^{3}H]GTN and [^{3}H]metabolite (Fig. 1B).

et al. (1981), i.e. 0.80 ± 0.5. Such a ratio indicates that GTN is not concentrated in the red blood cells. The B/P ratio of total metabolites was initially higher (1.22 ± 0.05) but quickly decreased and remained relatively constant (about 0.9).

The compositions of the 120-min blood incubation ether extracts (metabolites) as determined by HPLC are shown in Table 1. A sample chromatogram is shown in Fig. 2. These HPLC conditions will res lve GTN (2.4 min), 1,3-GDN (4.0 min), 1,2-GDN (6.1 min), 1-GMN (13.4 min) and 2-GMN (17.5 min). Note from Table 1 that all four GTN metabolites were present and that nearly all of the tritium label could be accounted for as these metabolites. It is also of some interest that the relative amounts of these metabolites were similar over an 8-fold concentration range.

The control experiments in which [^{3}H]GTN was incubated with buffer clearly indicate that the degradation of GTN is not due to hydrolysis at physiological pH values. Table 3 shows that [^{3}H]GTN is essentially stable for 21 h at 37°C. Incuba-

TABLE I

SUMMARY OF IN VITRO METABOLISM OF GTN BY HUMAN BLOOD

Initial GTN conc.	Subject	Percent of radioactivity accounted for in ether extract [a]										GTN $t_{1/2}$ (min)	GTN [b] B/P Ratio
		% 1,3-GDN		% 1,2-GDN		% 1-GMN		% 2-GMN		Total			
		Plasma	Blood	Plasma	Blood	Plasma	Blood	Plasma	Blood	Plasma	Blood		
	no. 1	25.4	28.1	49.7	45.8	4.0	6.4	18.3	25.9	97.4	106.3	28	0.66 ± 0.02
	no. 2	18.5	16.9	60.8	51.0	3.9	4.8	26.6	19.6	109.8	92.3	32	0.66 ± 0.03
136 ng/ml	no. 3	14.1	13.6	48.1	44.0	6.3	7.1	24.1	26.3	92.6	91.0	22	0.62 ± 0.05
	$\bar{X}$	19.3	19.5	52.9	46.9	4.7	6.1	23.0	23.9	99.9	96.5	27	-
	± S.D.	± 5.7	± 7.6	± 6.9	± 3.6	± 1.4	± 1.2	± 4.3	± 3.8	± 8.9	± 8.5	± 5	-
17 ng/ml	no. 1	11.5	-	52.9	-	5.1	-	23.3	-	92.8	-	15	0.65 ± 0.11

Recoveries of metabolites (mass balance) are shown in blood and plasma ether extracts. GTN half-lives and blood/plasma (B/P) ratios were determined between 0 and 60 min.

[a] % in ether extract at 120 min.

[b] ± S.D., n = 7, in 0–60 min measurements.

TABLE 2

INDIVIDUAL AND MEAN BLOOD–PLASMA RATIOS OF GTN AND TOTAL METABOLITES DETERMINED AT VARIOUS INCUBATION TIMES

Time (min)	136 ng/ml initial GTN concentration								17 ng/ml initial GTN concentration	
	GTN				Metabolites				GTN	Metabolites
	Subject 1	Subject 2	Subject 3	Mean ± S.D.	Subject 1	Subject 2	Subject 3	Mean ± S.D.	Subject 1	Subject 1
0	0.68	0.68	0.63	0.66 ± 0.03	1.27	1.18	1.20	1.22 ± 0.05	0.61	0.93
10	0.67	0.68	0.66	0.67 ± 0.01	1.04	0.98	0.99	1.00 ± 0.03	0.65	0.76
20	0.66	0.69	0.68	0.68 ± 0.02	1.09	0.91	0.80	0.93 ± 0.15	0.64	0.80
30	0.66	0.68	0.68	0.67 ± 0.01	1.06	0.88	0.88	0.94 ± 0.10	0.63	0.79
40	0.63	0.63	0.57	0.61 ± 0.03	0.96	1.01	0.93	0.97 ± 0.04	0.53	0.84
50	0.65	0.64	0.58	0.62 ± 0.04	0.93	0.96	0.89	0.93 ± 0.04	0.61	0.82
60	0.64	0.63	0.56	0.61 ± 0.04	0.85	0.90	0.92	0.89 ± 0.04	0.87	(a)
90	0.55	0.56	0.52	0.54 ± 0.02	0.89	0.91	0.95	0.92 ± 0.03	0.76	0.79
120	0.57	0.54	0.52	0.54 ± 0.03	0.91	0.89	0.92	0.91 ± 0.02	0.58	0.75

[^{3}H]GTN (136 or 17 ng/ml) was incubated at 37°C with fresh human blood.

[a] Sample lost during analysis.

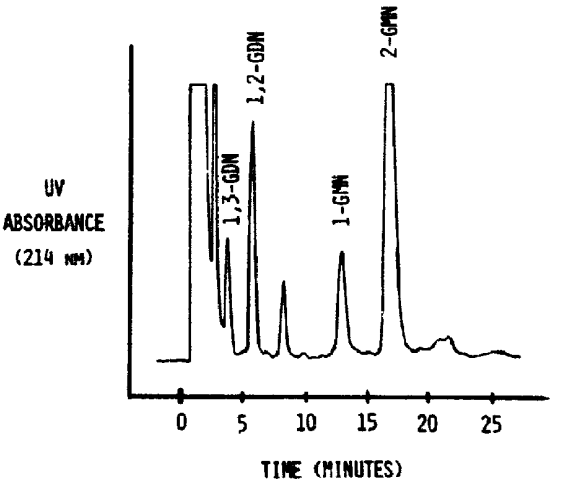

Fig. 2. HPLC chromatogram of a plasma ether extract containing 1,2-GDN, 1,3-GDN, 1-GMN and 2-GMN.

tion of [^{3}H]GTN with pooled human plasma, on the other hand, resulted in a first-order loss of GTN. The loss of GTN and accumulation of metabolites is shown in Fig. 3. [^{3}H]GTN was metabolized significantly slower in plasma than in blood. The mean plasma half-life was 202 ± 5 (S.D.) min. The rate of metabolism was 7.5-fold slower in plasma than blood. Armstrong et al. (1980) reported a similar observation, that plasma metabolism was 8.6-fold slower than blood.

The composition of plasma after incubation with [^{3}H]GTN for 21 h is shown in Table 4. Again, note that all of the radioactivity (tritium label) could be accounted for as the mono- and dinitrates. In fact, 1,3-GDN and 1,2-GDN account for 94% of the total plasma metabolites.

It is unclear why Wu et al. (1981) were unable to detect the GDNs after GTN incubation with blood. Several differences do exist between these two studies: (1) Wu et al. used cells from both fresh and outdated blood; (2) they used a one-step ethyl acetate extraction; and (3) a GC assay to detect GTN and GDNs. In the

TABLE 3

CONCENTRATIONS OF EXTRACTABLE [^{3}H]GTN AFTER INCUBATION WITH pH 7.4 BUFFER

Incubation time (h)	Concentration (ng/ml)	
	Pentane extract	Ether extract
0	130±6 [a]	8.0±0.2
2	136±3	8.2±0.2
21	132±7	8.3±0.5

Note that there are no significant changes in extractable [^{3}H]GTN levels over 21 hours.
[a] ±S.D. (n = 3).

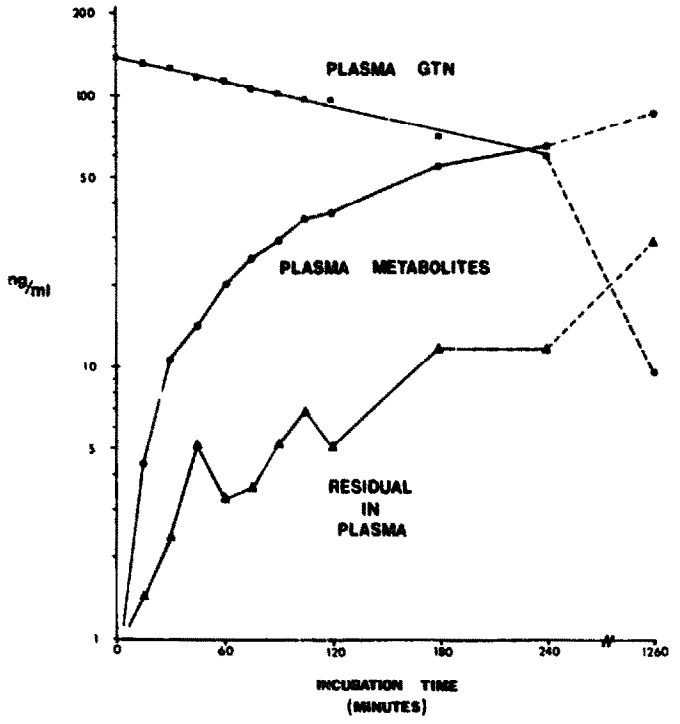

Fig. 3. In vitro metabolism of [^{3}H]GTN to [^{3}H]metabolites by human plasma. Figure shows the loss of [^{3}H]GTN (■), the accumulation of [^{3}H]metabolites (●) and the residual concentration (▲) after extractions. The data shown were normalized as in Fig. 1B.

present study, fresh blood was used. Armstrong et al. (1980), however, did not find a significant difference in the GTN half-life between whole blood and resuspended cells. In the present study, a two-step extraction with pentane and ether was used to remove GTN and metabolites. The single ethyl acetate extraction by Wu et al. should remove all of the GDNs and 80% of the GMNs, yet the GDNs were not

TABLE 4

METABOLITE COMPOSITION OF PLASMA AFTER INCUBATION OF [^{3}H]GTN (136 ng/ml) WITH WHOLE BLOOD OR POOLED PLASMA

	Metabolite composition (%)	
Metabolite	Blood incubation	Plasma incubation
1,3-GDN	19.3 ± 5.7 [a]	62.5 ± 5.4
1,2-GDN	52.9 ± 6.9	31.5 ± 0.5
1-GMN	4.7 ± 1.4	3.1 ± 1.0
2-GMN	23.0 ± 4.3	2.9 ± 3.2
Total	99.9 ± 8.9	100.1 ± 8.3

[a] ± S.D., n = 3.

detected. Therefore, neither the blood source nor the difference in solvents can account for the lack of metabolites. The final difference was the use of [^{3}H]GTN which allows the drug to be followed whether eliminated by metabolism or by some other 'physical loss.' The use of [^{3}H]GTN together with HPLC allowed the detection and quantitation of [^{3}H]GTN metabolites. Also, using [^{3}H]GTN, a mass balance was possible; 90–100% of the tritium label could be accounted for as the four GTN metabolites (Table 4).

In summary, the loss of GTN during in vitro incubation in blood was shown to be metabolic. Levels of [^{3}H]GTN were found to decrease at a pseudo-first-order rate (log-linearly) with half-lives ranging from 17 to 32 min. The half-lives varied with the blood source and the initial GTN concentration. Incubation of [^{3}H]GTN in plasma resulted in a much slower rate of metabolism (half-life = 202 ± 5 min). However, no degradation of [^{3}H]GTN could be detected after incubation in pH 7.4 buffer for 21 h. Thus, GTN degradation requires other cofactors present in blood and plasma. The decrease of GTN was accompanied by a simultaneous increase in metabolite levels. All four GTN metabolites (1,3-GDN, 1,2-GDN, 1-GMN and 2-GMN) were detected and quantitated after incubation in whole blood and plasma.

Acknowledgements

Supported in part by Center Grant GM 26691 and by a grant from Key Pharmaceuticals, Miami, FL. During the course of this work, P.K.N. received support as an AFPE James F. Hoge Memorial Fellow.

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