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EXAMINATION OF THE IN VITRO DEGRADATION OF [^{14}C]PENTAERYTHRITOL TETRANITRATE IN RAT AND HUMAN BLOOD WITH AN IMPROVED THIN-LAYER RADIOCHROMATOGRAPHIC PROCEDURE

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SUMMARY

Improvements were made on a reported thin-layer radiochromatographic assay for the determination of [^{14}C]pentaerythritol tetranitrate (PETN) and its metabolites in whole blood, using methanol instead of dioxane as the extracting solvent. Recovery of total radioactivity for the entire work-up procedure was greater than 90%, and the distribution of PETN and its metabolites in degraded blood samples was found to be reproducible. This modified method appeared simpler and yielded better recovery of radioactivity than the literature method. In vitro metabolism of [^{14}C]PETN in rat and human blood was examined by incubation of the drug with fresh blood at 37°C for 60 min. In rat blood, the half-life of PETN degradation was about 15 min producing the trinitrate, dinitrate and mononitrate metabolites. Human blood was also capable of degrading PETN in vitro, but at a lower rate than rat blood, yielding only the trinitrate metabolite in quantifiable amounts during the incubation period. Equilibrium of PETN between plasma and red blood cells was observed within 1 min after PETN addition to both rat and human blood. The apparent plasma/red blood cells partition ratios of PETN were 1.1 and 1.7 for rat and human blood, respectively. PETN degradation was approximately ten times slower in rat plasma than in rat blood, suggesting that enzymes in erythrocytes are important for PETN metabolism in rat whole blood.

INTRODUCTION

Pentaerythritol tetranitrate (PETN) has been shown to be an effective long-acting anti-anginal agent [1, 2]. DiCarlo et al. [3] showed that this organic nitrate is extensively metabolized and widely distributed into peripheral tissues in the rat. Systemic blood clearance of this drug in the rat was found to exceed

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the normal cardiac output [4]. In order to examine the contribution of various processes to the systemic blood clearance and to establish conditions for pharmacokinetic studies, it is necessary to assess the rate and extent of PETN degradation per se in rat whole blood. Literature reports have suggested that rat plasma [5], suspended red blood cells (RBC) in phosphate buffer [5], and human whole blood [6] are capable of degrading PETN enzymatically in vitro. However, quantitative information regarding the rates of PETN metabolism in rat and human blood, and its rate and extent of distribution between plasma and RBC, is not available from the literature.

The thin-layer radiochromatographic assay by DiCarlo and co-workers [3, 7, 8] for determining [^{14}C]PETN and its metabolites [i.e. pentaerythritol trinitrate (PE-tri-N), dinitrate (PEDN), mononitrate (PEMN) and polar metabolites] in whole blood was initially used in conducting these experiments. However, we found this procedure to have several drawbacks, viz. a tedious work-up procedure, relatively large sample size and poor total recovery of radioactivity (details to be described later). Improvements to this procedure appeared necessary.

In this report, we describe first the modifications we have made on the radiochemical chromatographic assay for the determination of PETN and metabolites. The in vitro distribution and metabolism of [^{14}C]PETN in rat and human blood were then examined with both the improved method and that of DiCarlo et al. [7].

MATERIALS AND METHODS

Materials

[^{14}C]1,2-PETN (1:7 in lactose, Warner-Lambert, Ann Arbor, MI, U.S.A.) with a specific activity of 1.71 mCi/mmol was used in this study. [^{14}C]PETN was extracted from this mixture with acetone, followed by solvent concentration and drug precipitation with addition of distilled water. The melting point (uncorrected) of the PETN collected was 138—140°C (lit. 140—142°C [9]). This purified PETN gave a single radioactive region on the thin-layer chromatographic (TLC) plate with R_F = 0.85—0.95, and had a radioactive purity of 97.1%. Unlabelled PETN (Warner-Lambert) was obtained with the same procedure and identified by TLC. All solvents used were at least reagent grade. All glassware was acid-washed and silanized prior to use.

Degradation of [^{14}C]PETN in rat blood

Pooled heparinized rat blood was obtained fresh before the incubation experiments via cannulae implanted in the carotid artery of five rats. A 10-ml aliquot of blood was pre-incubated with gentle stirring in a jacketed beaker for 10 min at 37°C, and gassed with oxygen—carbon dioxide (95:5). [^{14}C]PETN, dissolved in PEG-400, was spiked into blood to achieve initial PETN concentrations in blood of about 0.5 and 1.0 μg/ml. The volume of PEG-400 added to blood was either 50 or 100 μl. Aliquots of 1.0 ml blood were withdrawn at 1, 5, 10, 20, 40 and 60 min following PETN addition. A portion of this sample (0.3 ml) was assayed according to the modified dioxane procedure, as described later. The remaining sample (0.7 ml) was immediately centrifuged to separate

plasma from RBC. A 50-μl aliquot of either plasma or RBC was taken to measure total radioactivity. Another aliquot (200 μl) of plasma or RBC was then assayed according to the modified dioxane procedure. The hematocrit of pooled rat blood (44%) remained constant throughout the incubation period.

Degradation of [^{14}C]PETN in human blood

Fresh heparinized human blood was obtained from two volunteers, and pooled. Two initial PETN concentrations in blood, of about 0.4 and 1.0 μg/ml, were achieved by adding 50 and 100 μl of the drug solution prepared in PEG-400, respectively, and the same incubation and sampling procedures were used. A 200-μl aliquot of the blood sample was analyzed according to the methanol extraction procedure described below. The remaining sample (0.8 ml) was immediately centrifuged to separate plasma from RBC. A 50-μl aliquot of plasma or RBC was used to measure total radioactivity, and another aliquot (200 μl) of these two blood components was assayed according to the methanol extraction procedure. The hematocrit of pooled human blood (51%) also remained unchanged during the entire incubation period.

Degradation of [^{14}C]PETN in rat plasma

Pooled rat plasma was obtained from centrifugation of fresh pooled rat blood, which was collected through a cannula implanted in the carotid artery before the incubation experiment. The plasma stock solution (6.0 ml) was pre-incubated for 10 min at 37°C, and drug was added to it to achieve initial plasma concentrations of about 0.6 and 1.0 μg/ml. The volume of PEG-400 added was either 25 or 50 μl. Plasma samples (0.5 ml) were collected at 1, 10, 20, 30, 40 and 60 min after drug addition, and 25 μl of 0.1 *M* silver nitrate was added to precipitate proteins. Plasma sample was extracted with 2.0 ml of ethyl acetate four times, according to the procedure of Crew et al. [10]. The combined organic extract was concentrated under a stream of dry nitrogen at room temperature to about 200 μl for analysis with TLC. Recovery of radioactivity for the extraction and TLC separation procedure was 77.1 ± 6.6% (mean ± S.D., $n = 24$).

Modified dioxane procedure

The dioxane extraction procedure of DiCarlo et al. [7] was modified by omitting the extraction and evaporation steps with methanol and methanol—water (1:1). Each aliquot of blood, plasma or RBC was mixed with 10 vols. of 75% dioxane in water to precipitate proteins. The supernatant was collected, and the residue was washed three times with dioxane. The combined dioxane extracts were then concentrated under a stream of dry nitrogen at room temperature to about 200 μl, which was then applied onto a TLC plate. The plate was developed first using a reported solvent system [7] of toluene—ethyl acetate—1-butanol—water (10:5:2:2, upper phase), and then twice with a solvent system [7] of 1-butanol—ammonium hydroxide—water (4:1:3, upper phase) to separate all the radioactivity from interfering materials which remained at the origin of the TLC plate. After multiple (three times) developments, the silica gel region between R_F values of 0.2—1.0 was removed and

transferred from the plate to a test tube, and was then washed with 2.0 ml of methanol four times to extract radioactivity. The combined methanol extract was concentrated to about 200 μl for further TLC analysis.

Methanol extraction procedure

Each 200-μl aliquot of blood was first mixed with 2.0 ml of ice-cold methanol and 50 μl of 0.1 *M* silver nitrate. After collecting the supernatant, the residue was washed with 2.0 ml of methanol three times. The combined methanol extract was concentrated to about 200 μl for further TLC analysis.

Determination of total radioactivity in blood components

Total radioactivity in blood, RBC or plasma was determined with the following solubilization method [11]: a 50-μl aliquot of each blood component was mixed with 0.75 ml of Soluene-350 (Packard Instrument, Downers Grove, IL, U.S.A.) and 0.75 ml of isopropanol in a counting vial. An aliquot of 0.5 ml of 32% hydrogen peroxide was added to decolorize the sample following digestion of the sample at room temperature for 10 min. The mixture was then allowed to stand at room temperature for at least 2 h. Dimulume-30 (Packard Instrument), 15 ml, was added to the vial to prepare sample for liquid scintillation counting.

Thin-layer chromatography and radiochemical assay

Glass plates (5 × 20 cm) coated with silica gel (250 μm) were obtained from Brinkmann Instruments, (Westbury, NY, U.S.A.). The solvent system [7] consisted of toluene—ethyl acetate—1-butanol—water (10:5:2:2, upper phase). The developed region of 12 cm (origin to solvent front) was divided into twelve to twenty segments and scrapped off. The silica gel was then mixed with 10 ml of Dioxscint (National Diagnostics, Somerville, NJ, U.S.A.), and counted for 10 min by a Packard Tri-Carb Model 3255 liquid scintillation spectrometer (Packard Instrument). The counting efficiency of each sample was determined by using the external standard ratio or channels ratio method. An internal standard, [^{14}C]toluene (Packard Instrument), was also used to correct for quenching in samples with counting efficiency less than 60%.

RESULTS AND DISCUSSION

Refinement of TLC assay for PETN and metabolites

Fig. 1 shows a representative TLC profile in which the plate was divided into twenty regions. The recovered radioactivity shown at each region of the plate was the net counts per min (cpm) above background. It is clear that the chromatographic system adopted was well capable of separating PETN and its metabolites. The R_F values obtained in this study agreed well with the values reported in the literature (Table I). The polar metabolites probably include the pentaerythritol (PE) and glucuronide conjugates of PE-tri-N, PEDN and PEMN [10]. As the study progressed, we found it sufficient to divide the plates into twelve, rather than twenty, regions. In each case, the drug and metabolite regions were clearly separated by a region of only background radioactivity.

The TLC procedure of DiCarlo et al. [7] for the determination of PETN

and its metabolites in whole blood has several shortcomings. First, a relatively large blood sample size (i.e. 1.4—1.8 ml for rat blood) was required; this is not practical when serial samples of blood are taken for a metabolic study in a small animal like the rat. Second, the steps involving (a) uptake of radioactivity from the dry residue of dioxane extract using methanol, (b) removal of insoluble materials including lipids from sample with methanol—

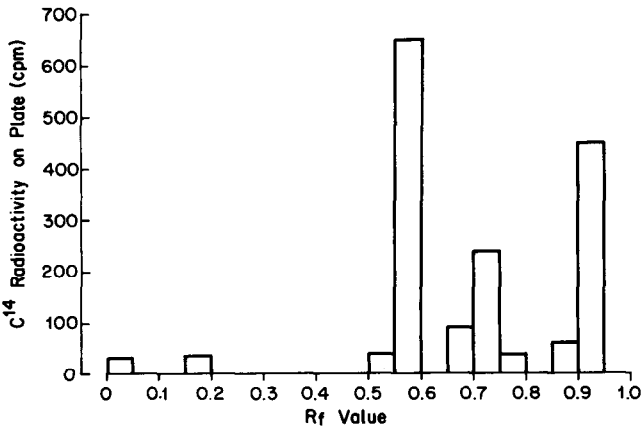


Fig. 1. Representative thin-layer chromatogram for separation of PETN and its metabolites. Solvent system: toluene—ethyl acetate—1-butanol—water (10:5:2:2, upper phase).

TABLE I

COMPARISON OF R_F VALUES FOR PETN AND ITS METABOLITES

Compound	R_F Value		
	DiCarlo et al. [3]	Carter et al. [12]	This study
PETN	0.77—0.84	0.93	0.85—0.95
PE-tri-N	0.60—0.69	0.74	0.65—0.80
PEDN	0.45—0.55	0.66	0.50—0.60
PEMN	0.16—0.23	0.29	0.15—0.25
Polar metabolites	0.0	0.0	0.0

TABLE II

COMPARISON OF RECOVERY OF RADIOACTIVITY FOR THE ENTIRE ASSAY PROCEDURE

Species	Method	Sample	Volume (ml)	Extracting solvent	Percentage recovery of radioactivity (mean ± S.D.)	Number of samples
Rat	DiCarlo et al. [8]	Blood	1.4—1.8	Dioxane	Not reported	Not known
	Modified dioxane procedure	Blood	0.3	Dioxane	77.8 ± 4.3	25
	Modified dioxane procedure	RBC	0.2	Dioxane	84.8 ± 12.6	25
	Modified dioxane procedure	Plasma	0.2	Dioxane	90.6 ± 9.8	25
	Methanol extraction procedure	Blood	0.5	Methanol	91.4 ± 1.1	4
Human	DiCarlo et al. [7]	Blood	8.0	Dioxane	Not greater than 60%	Not known
	Methanol extraction procedure	Blood	0.2	Methanol	92.2 ± 6.2	28
	Methanol extraction procedure	RBC	0.2	Methanol	87.5 ± 9.5	28
	Methanol extraction procedure	Plasma	0.2	Methanol	95.0 ± 4.6	28

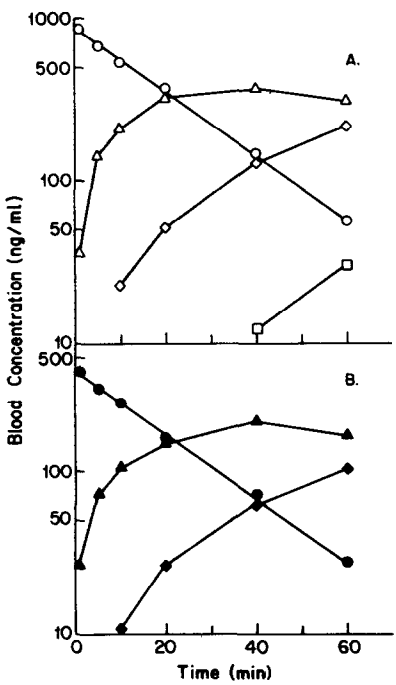
water (1:1) and (c) other steps before multiple developments of TLC plate were found unnecessary and therefore deleted. Third, the DiCarlo method [7] gave at most 60% recovery of radioactivity for human blood. In a preliminary experiment using a 0.5-ml sample of rat blood, we confirmed that recovery of radioactivity for this procedure was about 64%. This relatively poor recovery of radioactivity might be partly due to evaporative losses that occurred when the sample was taken to dryness. The modified dioxane procedure produced improved recovery (Table II), but it was still very tedious, primarily because of the long time required (9—10 h) to evaporate 8 ml of dioxane at room temperature. Since dioxane also extracted materials like lipids from blood which would interfere with the subsequent TLC separation, an additional 12-h multiple TLC development step was required.

The methanol extraction procedure utilized methanol in place of dioxane. Silver nitrate was added to aid the precipitation of proteins. In determining PETN and metabolites in rat whole blood, the methanol extraction procedure could recover about 91% of radioactivity during assay work-up (Table II). The relative percentages of radioactivity recovered as PETN, PE-tri-N, PEDN, PEMN and polar metabolites after incubation for 50 min in rat blood were reproducible and were 12.0 ± 0.4%, 50.9 ± 0.6%, 30.7 ± 0.6%, 5.43 ± 0.39% and 1.05 ± 0.06% (mean ± S.D., n = 4), respectively. Recovery and reproducibility were also found to be excellent with human blood, RBC and plasma samples (Table II).

It is important to determine the extent of degradation of intact PETN, if any, during assay work-up. Rat and human blood samples were assayed immediately after spiking with [^{14}C]PETN. The modified dioxane procedure gave PETN degradation of 13.9 ± 3.8% (mean ± S.D., n = 6) yielding PE-tri-N. The methanol extraction procedure also produced artifactual degradation of PETN to PE-tri-N in human blood during work-up to the extent of 11.1 ± 2.6% (n = 4). No further degradation of PE-tri-N was observed in either rat or human blood. In subsequent experiments, the experimentally determined concentrations of PETN in rat or human blood sample were corrected for this artifactual degradation.

In vitro metabolism of PETN in rat and human blood

The blood concentration versus time profiles of PETN and its metabolites obtained from in vitro incubation of [^{14}C]PETN in rat and human blood are shown in Figs. 2 and 3, respectively. PETN degradation in both rat and human blood appeared to follow apparent first-order kinetics for the two initial PETN concentrations examined. The half-lives of PETN degradation in rat and human blood are summarized in Table III. Degradation rate constant for PETN metabolism, k, was determined by non-weighted linear regression using all the concentration versus time data, and the half-life was calculated by $0.693/k$. PETN metabolism in rat blood was three to four times faster than that in human blood (Figs. 2 and 3). Human blood degraded PETN to PE-tri-N, but no further degradation of PE-tri-N was observed during the incubation period of 60 min. However, PETN and its three metabolites (i.e. PE-tri-N, PEDN and PEMN) were found in incubations with rat blood. An apparent concentration dependency was suggested for PETN metabolism in human blood but not in rat



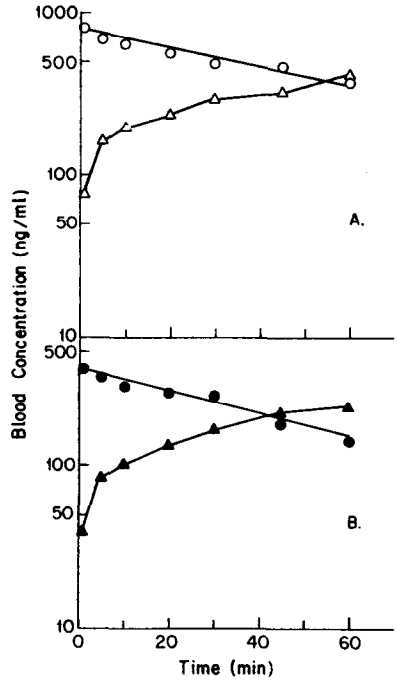


Fig. 2. Blood concentrations (average from duplicate runs) of PETN (○, ●), PE-tri-N (△, ▲), PEDN (◇, ◆) and PEMN (□, ■) during incubation in rat blood. (A) Initial concentration of PETN = 1.07 μg/ml; (B) initial concentration of PETN = 0.575 μg/ml. The difference between duplicate runs (expressed as the percentage of average value) had a 95% confidence limit of 6.1 to 14.2% with a median of 9.8% (n = 34).

Fig. 3. Blood concentrations (average from duplicate runs) of PETN (○, ●) and PE-tri-N (△, ▲) during incubation in human blood. (A) Initial concentration of PETN = 0.932 μg/ml; (B) initial concentration of PETN = 0.456 μg/ml. The difference between duplicate runs (expressed as the percentage of average value) had a 95% confidence limit of 8.2 and 19.8% with a median of 14.0% (n = 28).

TABLE III

IN VITRO DEGRADATION OF PETN METABOLISM IN RAT AND HUMAN WHOLE BLOOD

Species	Initial PETN concentration (μg/ml)	Half-life* (min)		
		Blood	RBC compartment**	Plasma compartment**
Rat	0.575	15.6 (15.2, 15.9)	14.8 (14.5, 15.0)	14.5 (13.3, 15.6)
	1.07	15.1 (12.9, 17.2)	14.6 (13.1, 16.1)	15.8 (13.7, 17.9)
Human	0.456	43.7 (35.4, 51.9)	48.3 (44.5, 52.1)	49.4 (44.5, 54.3)
	0.932	59.8 (53.4, 66.1)	68.0 (63.2, 73.7)	60.6 (53.0, 68.2)

*Average from duplicate runs; individual values are listed in parentheses.
**Degradation was conducted in whole blood.

blood (Table III). Since PETN has been shown to be stable in buffer solutions of pH 7.4 up to 6 h [6], degradation of PETN in rat or human blood was most likely due to enzyme-mediated processes.

The patterns of PETN decline and metabolite formation in the plasma and RBC components were similar to the respective pattern found in rat and human whole blood. Since the half-life of PETN degradation was similar for both plasma and RBC for the entire incubation, it would appear that distributional equilibrium was rapidly achieved. The apparent plasma/blood and plasma/RBC partition ratios of PETN in rat and human blood were, therefore, computed by using all the PETN concentration—time data (Table IV). Both plasma/RBC and plasma/blood ratios of PETN in rat blood were close to 1 indicating little preferential accumulation of PETN in RBC. In contrast, both plasma/RBC and plasma/blood ratios of PETN in human blood were significantly larger than unity ($p < 0.05$) suggesting reduced PETN distribution into RBC. It has been shown that nitrate reductase type of enzymes locate mainly in human RBC [13], thus the higher plasma/RBC PETN partition ratio in human blood might be part of the reason for the lower rate of degradation in human than in rat blood. However, it is also possible that the concentration and activity of nitrate reductase in erythrocytes may also be species-dependent.

In order to further assess the role of RBC on PETN metabolism in rat blood, degradation of PETN in separated rat plasma was examined. Fig. 4 shows the plasma concentration versus time profiles of PETN and PE-tri-N when two different initial PETN concentrations were used. PETN appeared to degrade about ten times slower in rat plasma than in rat blood. The half-lives of degradation of PETN in plasma were estimated roughly at 140 and 160 min for initial PETN concentrations of 0.68 and 0.96 μg/ml, respectively. No degradation of PE-tri-N was found in rat plasma during this 60-min incubation period. These results support the conclusion that enzymes in erythrocytes were primarily responsible for the metabolism of PETN in rat whole blood.

In summary, an improved thin-layer radiochromatographic assay procedure was developed for the determination of PETN and its metabolites in rat and human blood. PETN was extensively metabolized in vitro by rat blood to yield PE-tri-N, PEDN and PEMN. Human blood could also metabolize PETN in vitro but at a lower rate than rat blood. The apparent plasma/RBC partition ratios of PETN were 1.1 and 1.7 for rat and human blood, respectively. Erythrocyte enzymes, rather than those in plasma, appeared to be mainly responsible for PETN metabolism in whole blood.

TABLE IV

VALUES OF APPARENT PARTITION RATIOS OF PETN IN RAT AND HUMAN BLOOD

Species	Initial PETN concentration (μg/ml)	Apparent partition ratio (mean ± S.D.)	
		C_{plasma}/C_{RBC}	C_{plasma}/C_{blood}
Rat (n = 12)	0.575	1.1 ± 0.2	0.95 ± 0.14
	1.07	1.1 ± 0.2	0.97 ± 0.16
Human (n = 14)	0.456	1.7 ± 0.2	1.3 ± 0.2
	0.932	1.6 ± 0.1	1.2 ± 0.1

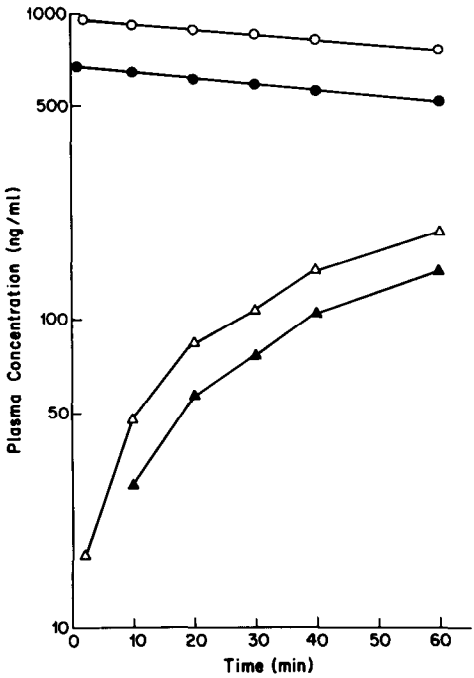


Fig. 4. Plasma concentrations (average from duplicate runs) of PETN (○, ●) and PE-tri-N (△, ▲) during incubation in rat plasma. Closed symbols: initial concentration of PETN = 0.68 μg/ml; open symbols: initial concentration of PETN = 0.96 μg/ml. The difference between duplicate runs (expressed as the percentage of average value) had a 95% confidence limit of 1.3 and 7.7% with a median of 5.0% (n = 24).

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