Biochemical Pharmacology, Vol. 17, pp. 2179–2183. Pergamon Press. 1968. Printed in Great Britain

THE ABSORPTION AND BIOTRANSFORMATION OF GLYCERYL TRINITRATE-1,3-^{14}C BY RATS

FREDERICK J. DICARLO, MALCOLM C. CREW, LLOYD J. HAYNES, MYRIAM D. MELGAR and ROSEMARIE L. GALA

Biochemistry Department, Warner-Lambert Research Institute, Morris Plains, N.J., U.S.A.

(*Received* 20 *March* 1968; *accepted* 3 *May* 1968)

Abstract—The metabolism of ^{14}C-labeled glyceryl trinitrate was studied in the rat. Absorption, tissue distribution and elimination were followed after administering a single dose by gavage. Thin-layer chromatography and radio-scanning methods were employed to assay urinary drug metabolites, including the isomeric glyceryl dinitrates and glyceryl monoitrates. It was found that glyceryl trinitrate was absorbed and transformed very rapidly. The major products were carbon dioxide, urinary glycerol, glyceryl nitrates, organic acids and some unidentified tissue components which were also labeled with ^{14}C.

GLYCERYL trinitrate (nitroglycerin) is a highly regarded antiranginal compound with an extremely rapid onset of activity. The first studies on the metabolism of this drug, published in 1883[1] and 1885,[2] disclosed that inorganic nitrite was formed *in vivo* and *in vitro*. Nitrite remained the only known metabolite of glyceryl trinitrate until 1964, when glyceryl dinitrates were reported to be formed by incubating the drug with hog liver.[3] Subsequently, the glyceryl dinitrates[4] and traces of glyceryl mononitrate[5] were detected in the urine of rats dosed orally or i.p. with glyceryl trinitrate. Additionally, it was reported that no glycerol was formed from glyceryl trinitrate either *in vivo* or *in vitro*.[4, 5] This incomplete degradation seemed to merit reinvestigation in view of the complete de-esterification by rats of pentaerythritol tetranitrate,[6] a compound which is far more resistant than glyceryl trinitrate to enzymatic transformation.[5] The previous reliance upon nitrate assays[3–5] failed to allow for the possible formation of glycerol from glyceryl trinitrate. In order to gain the capability of conveniently detecting glycerol, the present study was performed with ^{14}C-labeled glyceryl trinitrate.

MATERIALS AND METHODS

^{14}C-*glyceryl trinitrate.* The glyceryl trinitrate used in this study was synthesized from (1,3-^{14}C)-glycerol by the procedure of Lawrie.[7] Purification according to Dunstan *et al.*[8] yielded a product with 99·9 per cent radiochemical purity. In order to minimize the danger of explosion, the product was diluted with 19 parts by weight of chemically pure lactose. The sp. act. of the mixture was 0·18 mc/g.

Animals and dosing. Male CFN Wistar rats (Carworth Farms), each weighing about 200 g, were employed. A single dose (10 mg/kg) of ^{14}C-glyceryl trinitrate was administered by gavage to each rat. Then the animals were housed individually in Aerospace glass metabolic units without food or water. Groups of 3 rats were studied for experimental periods of 30 min, 1 hr, 2 hr and 4 hr.

Carbon dioxide. Three metabolic units were arranged so that the exhaled gases were drawn by suction through a train of 10 gas-washing bottles, each containing 80 ml of 5% NaOH.

Blood, tissues and excreta. At the end of each study period, blood samples were withdrawn by heart puncture and the animals were killed immediately for excision of the heart, lungs, liver, entire gastrointestinal tract, spleen and kidneys. These blood and tissue samples were pooled as were the remaining carcasses and the urine and stool collections.

Radioactivity counting. Quantitative assays were conducted with the use of a Packard Tri-Carb model 3003 liquid scintillation spectrometer. One ml of each urine collection was diluted with 18 ml of scintillation solution and counted directly. The scintillation solution consisted of 7·0 g of PPO (2,5-diphenyloxazole), 0·3 g of dimethyl POPOP (1,4-*bis*-2-[4-methyl-5-phenyloxazolyl]-benzene) and 100·0 g naphthalene in 1·0 liter of redistilled dioxane.

The $^{14}CO_2$ assay was carried out by gaseous diffusion from acidified NaOH solution into hyamine hydroxide, which was subsequently dissolved in scintillation fluid for counting. Two aliquots (1·0 and 2·0 ml) were taken from the NaOH solution representing a given collection period. Each aliquot was placed into a 25-ml Erlenmeyer flask and closed with a rubber septum (Kontes) fitted with a polyethylene center well (Kontes) containing 0·2 ml of a 1 M solution of hyamine hydroxide in methanol (Packard). The NaOH solution in the flask was acidified by injecting 1·0 ml of 6 N H_2SO_4 through the septum with a hypodermic syringe. After 18 hr, the center well was removed from the flask and placed into a vial containing 18 ml of scintillation fluid for counting.

Each blood pool was diluted 50-fold with distilled water and 1·0 ml aliquots were mixed with 18 ml of scintillation solution for counting.

The tissue, carcass and feces pools were homogenized in a Waring–blendor with 75% dioxane and filtered. The filtration residues were re-extracted twice in the same manner, and the pooled extracts were assayed for ^{14}C by scintillation spectrometry. Radioactivity remaining in the extracted residues was determined by digesting 10-mg portions of these residues with 1·5 ml hyamine 10X (1 M in methanol) and 0·5 ml water for 24 hr at 37°. Then the entire digestion mixtures were counted by scintillation spectrometry.

Thin-layer chromatography. TLC's were developed by the ascending method on 2 × 8 in. plates coated with 250 μ silica gel G bound with calcium sulfate. The solvents used were: 106, 1-butanol:acetic acid:water (5:1:4, v/v/v); 204, benzene-ethyl acetate: acetic acid (16:4:1, v/v/v); and 402, ethyl acetate:*n*-heptane (9:1, v/v). Solvents 106 and 204 were used with chamber saturation (filter paper lining the side of the cylinder). However, solvent 402 produced well defined resolution of glyceryl mononitrates only without chamber saturation. The developed chromatograms were scanned with a Packard model 7200 radiochromatogram scanner to determine the R_f values of the radioactive bands. The area under each peak was measured with a planimeter in order to determine the relative quantity of each labeled component in the sample.

^{14}C-labeled metabolites in urine. Preliminary experiments showed that the labeled acidic components of urine were retained by Amberlite IR4B resin (OH^- form) provided that the urine was first percolated through a column of Dowex 50W-X2 resin (H^+ form). These tests also showed that glycerol and glyceryl trinitrate were unaltered by passage through these ion-exchangers.

Aliquots of the diluted urine containing 0·05 to 0·2 μc of ^{14}C were percolated through a column prepared from 1·0 g of the Dowex resin placed over 1·5 g of the Amberlite resin. Each neutral effluent was collected and monitored until the radioactivity fell to an insignificant level. Approximately three times the volume of the original sample was collected. The acids retained by the resin were then flushed from the column with 3 N NH_4OH in order to confirm that all of the radioactivity in the sample had been recovered.

The neutral effluent was concentrated without loss of radioactivity by evaporation to a volume of 2 ml. Thin-layer chromatograms were run on the concentrated neutral fraction in order to determine the proportions of glycerol, the glyceryl nitrates and other neutral compounds present. The developing solvents used were as described above.

RESULTS

Absorption. The data in Table 1 illustrate the rapid absorption of ^{14}C-glyceryl trinitrate. This is especially clear from the observation that more than half of the

TABLE 1. DISTRIBUTION OF ^{14}C AFTER ORAL ADMINISTRATION OF ^{14}C-GLYCERYL TRINITRATE TO RATS

	Per cent of administered radioactivity found after							
	0·5 hr		1 hr		2 hr		4 hr	
Specimen	Extract	Residue	Extract	Residue	Extract	Residue	Extract	Residue
Blood*	6·36		5·34		3·16		1·88	
GI tract	42·28	3·41	32·34	5·74	24·05	4·98	16·42	5·05
Heart	0·12	0·09	0·13	0·01	0·06	0·07	0·05	0·05
Kidney	0·40	0·08	0·30	0·38	0·16	0·34	0·12	0·17
Liver	2·33	5·00	1·74	4·90	1·48	3·52	0·70	1·83
Lung	0·13	0·16	0·16	0·12	0·09	0·10	0·07	0·08
Spleen	0·06	0·01	0·06	0·04	0·03	0·08	0·02	0·02
Carcass	21·78	10·52	19·01	9·45	11·22	5·97	9·59	7·04
Urine	2·91		6·13		15·51		20·95	
Feces	0·03	0·02	0·72	0·11	0·14	0·09	1·65	0·62
CO_2	2·42		7·31		13·18		19·76	
Total recovery	98·11		93·99		84·23		86·07	

* Blood was assumed to constitute 10% of body weight.

radioactivity was removed from the gastrointestinal tract within 30 min after drug administration. The remarkably high blood level at this time, amounting to 6·4 per cent of the dose, also manifests the high absorption rate. Although this blood level represented the greatest observed concentration, the actual maximum might have occurred earlier.

Tissue distribution. The major tissue concentrations of absorbed ^{14}C were found in the liver and in the carcass. The liver held 7·3 per cent of the administered ^{14}C at 30 min, but only 2·5 per cent after 4 hr. The carcass radioactivity decreased from 33 per cent to about half that amount over the same interval. The heart, lung, kidney and spleen took up small quantities of ^{14}C from labeled glyceryl trinitrate and the concentrations decreased over the period investigated.

Elimination. Radioactivity from ^{14}C-glyceryl trinitrate quickly appeared in the urine and in the exhaled gases (Table 1). The quantity of radioactivity eliminated as $^{14}CO_2$ kept pace with the ^{14}C excreted into the urine. The passage of radioactive compounds into the feces proceeded relatively slowly, and at the end of 4 hr amounted to only about 2·2 per cent of the dose as compared with roughly 20 per cent each for the urine and for the respiratory carbon dioxide.

Anabolism. As time passed, increasing proportions of the total radioactivity were found to resist extraction from the gastrointestinal tract and carcass (Table 1). The percentage of radioactivity not extracted from the gastrointestinal tract increased steadily from about 7 per cent at 30 min to almost 25 per cent at 4 hr. Over the same period, the residual ^{14}C in the carcass rose from about 33 to 42 per cent of the total radioactivity. The kidney data followed a similar pattern and at 2 and 4 hr contained more residual than extractable radioactivity. All of the liver samples contained more residual than extractable radioactivity and the proportion of these two components remained quite constant throughout the experimental period. The heart and lung specimens contained approximately the same quantity of radioactivity as percentage of dose, and in most instances the ^{14}C was about equally divided between the extractable and residual fractions. The feces also showed the presence of labeled compounds insoluble in dioxane. Since glycerol and all of its nitrates are readily soluble in dioxane, the residual radioactivity is ascribed to anabolic products formed from glycerol. The presence of bound glyceryl nitrates in the residues may be discounted, because even the less readily extractable nitrates of pentaerythritol are easily removed from rat tissues with dioxane.[6]

Catabolism. The extensive conversion of ^{14}C-glyceryl trinitrate to $^{14}CO_2$ is assumed to be preceded by complete de-esterification to glycerol. No unchanged drug was found in any urine sample (Table 2). The principal metabolite in all of the collections

TABLE 2. QUALITATIVE AND QUANTITATIVE ASSAY OF ^{14}C COMPOUNDS IN RAT URINE AFTER ^{14}C-GLYCERYL TRINITRATE ADMINISTRATION

Metabolite	Per cent of administered ^{14}C			
	30 min	1 hr	2 hr	4 hr
Glyceryl-1,2-dinitrate	0·40	0·10	0·87	1·28
Glyceryl-1,3-dinitrate	0·11	0·04	0·43	0·51
Glyceryl-1-nitrate	0·20	0·25	2·05	2·41
Glyceryl-2-nitrate	0·23	0·16	1·62	1·88
Glycerol	0·95	2·80	4·25	6·64
R_f 0·00 in solvent 106			0·61	0·70
R_f 0·16 in solvent 106		0·22	0·78	0·81
R_f 0·34 in solvent 106	0·21	0·85	1·65	2·63
Acids	0·81	1·71	3·25	4·09
Total	2·91	6·13	15·51	20·95

was glycerol. Second in abundance were organic acids; these radioactive compounds were not further identified. Both glyceryl dinitrate isomers were present in all of the urine samples. The concentration of glyceryl-1,2-dinitrate was always greater than that of the 1,3-dinitrate, and in 3 of the 4 urine collections, there was approximately twice as much of the 1,2-isomer. Both glyceryl monoitrates were also present in all urine

samples, usually with the glyceryl-1-nitrate in preponderance. Three unidentified radioactive areas were detected chromatographically in some urine samples. These areas probably represent neutral or amphoteric compounds. One of them (R_f 0·34 in solvent 106) increased steadily with time and eventually represented more than 12 per cent of the urinary radioactivity.

DISCUSSION

The oral administration of glyceryl trinitrate to rats was followed by rapid absorption, metabolism and excretion. The drug was de-esterified stepwise with no apparent major initial preference for either the primary or the secondary nitrate group. This conclusion is based upon the observation that the urine usually contained about twice as much glyceryl-1,2-dinitrate as glyceryl-1,3-dinitrate. Needleman and Hunter[5] actually obtained approximately the same result by incubating glyceryl trinitrate with hepatic organic nitrate reductase. However, this finding is not clear from their publication[5] because the opposite structures were assigned to glyceryl-1,2-dinitrate and glyceryl-1,3-dinitrate. This confusion of the isomers resulted from reliance upon methodology published in 1908.[9] Recently, the glyceryl dinitrates were identified unequivocally[10] on the basis that partial hydrolysis can convert glyceryl-1,3-dinitrate to only a single product (glyceryl-1-nitrate), whereas glyceryl-1,2-dinitrate yields two partially de-esterified products (glyceryl-1-nitrate and glyceryl-2-nitrate).

One of the drug metabolites was glycerol and it would obviously enter into normal metabolic processes. The radioactive tissue components which resisted extraction with dioxane may include glycogen[11–13] and a variety of lipids.[12, 13] Until considerably more information is developed in this connection, ^{14}C-labeled glyceryl trinitrate should not be administered to humans.

Acknowledgement—The authors appreciate the cooperation of Mr. Edward J. Merrill of this Institute and Dr. Frank D. Pickel of Evans Research and Development Corp., who were responsible for the synthesis of the radioactive nitroglycerin used in this study.

REFERENCES

1. M. Hay, *Practitioner* **30**, 422 (1883).
2. M. Cagnoli, *Jber. Fortschr. Tierchem.* **15**, 92 (1885).
3. P. Needleman and J. C. Krantz, Jr., *Fedn Proc.* **23**, 178 (1964).
4. P. Needleman and J. C. Krantz, Jr., *Biochem. Pharmac.* **14**, 1225 (1965).
5. P. Needleman and F. E. Hunter, Jr., *Molec. Pharmac.* **1**, 77 (1965).
6. F. J. DiCarlo, M. C. Crew, C. B. Coutinho, L. J. Haynes and N. J. Sklow, *Biochem. Pharmac.* **16**, 309 (1967).
7. J. W. Lawrie, *Glycerol and the Glycols*, p. 318. Chemical Catalog Co., New York (1928).
8. I. Dunstan, J. V. Griffiths and S. A. Harvey, *J. Chem. Soc.* 1319, 1325 (1965).
9. W. Will, *Ber. dt. chem. Ges.* **41**, 1107 (1908).
10. M. C. Crew and F. J. DiCarlo, *J. Chromat.*, **5**, 506 (1968).
11. L. F. Catron and H. B. Lewis, *J. biol. Chem.* **84**, 553 (1929).
12. A. P. Doerschuk, *J. biol. Chem.* **193**, 39 (1951).
13. L. I. Gidez and M. L. Karnovsky, *J. biol. Chem.* **206**, 229 (1954).