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IDENTIFICATION AND ASSAY OF ISOMERIC ^{14}C-GLYCERYL NITRATES

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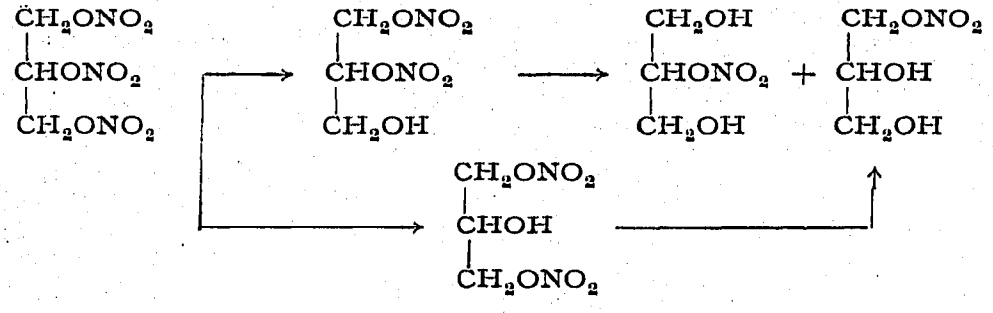
SUMMARY

A method was developed for the differentiation of glyceryl-1,2-dinitrate from glyceryl-1,3-dinitrate and glyceryl-1-nitrate from glyceryl-2-nitrate. Using thin-layer chromatography and ^{14}C labeling, these compounds can be assayed quantitatively and simultaneously in the presence of glyceryl trinitrate and glycerol.

Although the lower nitrates of glycerol have been known since 1908[1], their absolute identification remained in doubt until 1965[2]. Despite this recent clarification, it was difficult to distinguish and to estimate the two glyceryl dinitrates and the two glyceryl mononitrates in mixtures of all four compounds. This problem becomes especially acute when dealing with systems in which glycerol and glyceryl trinitrate may also be present. We addressed our attention to the assay of the isomeric glyceryl nitrates in order to cope with the metabolites expected in the urine of animals dosed with ^{14}C-glyceryl trinitrate. The differentiation of the isomeric dinitrates was based upon the fact that glyceryl-1,3-dinitrate can yield only a single mononitrate upon partial hydrolysis whereas glyceryl-1,2-dinitrate can yield glyceryl-1-nitrate and glyceryl-2-nitrate. Distinguishing the dinitrates could serve to identify the mononitrates as well because glyceryl-1,3-dinitrate can yield only glyceryl-1-nitrate.



EXPERIMENTAL

Thin-layer chromatography

Thin-layer chromatograms were run on 2 × 8 in. glass plates coated with Silica Gel G to a thickness of 250 μ. The chromatograms were developed by the ascending

method to a height of 10 cm above the origin unless stated otherwise. The solvents employed were:

105: 1-butanol–conc. NH_4OH–water (4:1:3, v/v/v)
106: 1-butanol–acetic acid–water (5:1:4, v/v/v)
201: toluene–ethyl acetate (1:1, v/v)
203: toluene–ethyl acetate (4:1, v/v)
204: benzene–ethyl acetate–acetic acid (16:4:1, v/v/v)
401: ethyl acetate saturated with water, and
402: ethyl acetate–*n*-heptane (9:1, v/v).

All solvents were used with chamber saturation (filter paper lining the side of the cylinder) with the exception of solvent 402 which produced clear cut resolution of the mononitrates only without saturation.

The chromatograms were scanned with a Packard Model 7201 Radiochromatogram Scanner to determine the R_F values of the radioactive bands. Peak areas on the scans were measured with a planimeter to determine sample composition.

Autoradiograms were prepared by exposure of the thin-layer plates containing approximately 0.03 μC of ^{14}C to X-ray film for 24 h.

Radioactive glyceryl trinitrate

[1,3-^{14}C]-Glyceryl trinitrate was synthesized at Evans Research and Development Corp. from [1,3-^{14}C]-glycerol by the nitration procedure of LAWRIE[3]. Purification according to DUNSTAN *et al.*[2] yielded a product with 99.9 % radiochemical purity. In order to minimize the danger of explosion, the product was diluted with 19 parts by weight of C.P. lactose. The specific activity of the mixture was 0.18 mC/g.

Radioactive glyceryl dinitrates

The source of ^{14}C-glyceryl dinitrates was crude ^{14}C-glyceryl trinitrate from a preliminary nitration experiment. The crude material was contained in a slurry with 50 ml of 30 % ethanol and 18 g of lactose. In order to remove glyceryl trinitrate, the slurry (845 μC, sp.ac. 275 μC/mmole) was dissolved in 50 ml of 20 % methanol and extracted 30 times with 25 ml portions of *n*-heptane. Each heptane extract was washed with the same 5 ml of 20 % methanol before pooling. The heptane pool was extracted with one 50 ml portion and two 25 ml portions of 95 % methanol as a means of concentrating the radioactive glyceryl trinitrate.

Ether was used to extract ^{14}C-glyceryl dinitrates from the combination of the raffinate from the heptane extractions and the 5 ml of methanol wash. After dilution with water to 100 ml, eight extractions were performed with 25 ml volumes of ether. Separate pools were made of the first four and the second four extracts.

The aqueous solution was treated with 50 g of ammonium sulfate (0.5 g/ml) and extracted with eight 25 ml portions of ether–ethanol (3:1, v/v). Separate pools were made of the first four and of the second four extracts.

All of the fractions were assayed by scintillation counting and by thin-layer chromatography in solvent 204.

The two glyceryl dinitrates were separated from the first ether extract fraction by thin-layer chromatography. Aliquots (0.2 ml) containing 0.35 μC of ^{14}C each were spotted on 20 thin-layer plates and developed in solvent 204 to a height of 15 cm.

Each plate was scanned to locate the radioactivity which was then scraped off the plate in such a manner as to leave as wide a space as practicable between the two dinitrates. The dinitrates were eluted with ether from the two portions of silica gel at R_F 0.36 and 0.47.

Nitrate assays

The three main fractions (heptane, first ether extract, first ethanol–ether extract) from the fractionation of crude glyceryl trinitrate were assayed for nitrate using phenoldisulfonic acid[4]. Appropriate aliquots of each fraction were evaporated in Coleman cuvettes (19 × 105 mm) and dissolved in 0.2 ml of glacial acetic acid. Standards containing 10–50 μg of nitrate (as potassium nitrate) in 0.2 ml of glacial acetic acid were run simultaneously. Each sample was treated with 0.2 ml of phenoldisulfonic acid solution (APHA-Harleco). After 10 min at room temperature the samples were diluted with 2.0 ml of water, chilled in ice, and diluted successively with 2.0 ml of conc. NH_4OH and 4.0 ml of water. The color developed was read at 410 mμ in a Coleman Jr. spectrophotometer against a reagent blank. The nitrate content of each tube was determined from the standard curve. The glycerol content of each tube was determined by scintillation counting; a 0.10 ml aliquot of the total 8.1 ml cuvette content was diluted with 1.0 ml of methanol to increase the solubility of the mixture in the scintillation solvent (dioxane).

Hydrolysis of glyceryl trinitrates

The stability of glyceryl trinitrate in aqueous solutions was evaluated by hydrolysis at 37° in NaOH, water and HCl.

A 0.5 mg sample of ^{14}C-glyceryl trinitrate (1.8 μC) in 9.5 mg of C.P. lactose was dissolved in 5 ml of 4 *N* NaOH and placed in a bath at 37°. After 15 min a 0.2 ml aliquot of the solution was added to a chilled (0°) 0.2 ml portion of 5 *N* acetic acid and assayed immediately by thin-layer chromatography in solvent 204. No aliquots from later time intervals were examined.

A 0.5 mg sample of ^{14}C-glyceryl trinitrate (1.8 μC) in 9.5 mg of C.P. lactose was dissolved in 5 ml of water and placed in a bath at 37°. After 6 h an aliquot was assayed directly by thin-layer chromatography in solvent 204. No further aliquots were examined.

A 0.5 mg sample of ^{14}C-glyceryl trinitrate in 9.5 mg of C.P. lactose was dissolved in 5 ml of 4 *N* HCl and placed in a bath at 37°. Aliquots (0.2 ml) were removed at 6, 24 and 48 h, added to chilled 0.2 ml portions of 5 *N* NH_4OH and assayed immediately by thin-layer chromatography in solvent 204.

Hydrolysis of glyceryl dinitrates

The ether was evaporated from the separated dinitrates which were then dissolved in 1.0 ml of 4 *N* HCl. After 24 h at 37° the two mixtures were neutralized with NH_4OH and concentrated by evaporation for thin-layer chromatography in several solvent systems. In addition to radioscanning, the plates were autoradiographed in order to detect the separation of bands not resolved by the radioscanner.

RESULTS

The data presented in Table I show that the crude ^{14}C-glyceryl trinitrate consisted of approximately 77 % trinitrate, 19 % dinitrates, 3 % mononitrates and 1 %

TABLE I

PREPARATION OF A DINITRATE-RICH FRACTION FROM CRUDE ^{14}C-GLYCERYL TRINITRATE

Fraction	*% of total ^{14}C present as*				*% ^{14}C in fraction*
	Glycerol	*Glyceryl mononitrate*	*Glyceryl dinitrate*	*Glyceryl trinitrate*	
Heptane extract				76.20	76.20
1st Ether extract		1.62	18.28	0.81	20.71
2nd Ether extract		0.48	0.62		1.10
1st Ether–ethanol extract	0.18	0.73			0.91
2nd Ether–ethanol extract	0.15	0.21			0.36
Residue	0.73				0.73
Total	1.06	3.04	18.90	77.01	100.0

glycerol. The tedious heptane extraction from aqueous methanol removed 99 % of the glyceryl trinitrate present without detectable contaminants. The subsequent extractions with ether completely removed glyceryl dinitrates. The first ether extract was the best suited to differentiating glyceryl nitrate isomers. This fraction consisted of 88.7 % dinitrates, 3.9 % trinitrate and 7.8 % mononitrates.

Table II shows the results obtained by assaying the various fractions for glycerol and for nitrate. In all instances, the found nitrate/glycerol ratio agrees with the ratio calculated from the data in Table I.

TABLE II

ASSAY OF MAJOR FRACTIONS PREPARED FROM CRUDE ^{14}C-GLYCERYL TRINITRATE

Fraction	*Sample assayed (nC)*	*nmoles found*		*Molar ratio, nitrate/glycerol*	
		Glycerol	*Nitrate*	*Found*	*Calculated**
Heptane extract	30.1	110	326	2.96	3.00
Heptane extract	60.7	221	667	3.02	3.00
1st Ether extract	27.9	102	200	1.96	1.96
1st Ether extract	58.9	214	397	1.86	1.96
1st Ether–ethanol extract	38.8	141	119	0.84	0.81
1st Ether–ethanol extract	95.8	349	312	0.89	0.81

* Based upon composition of fractions in Table I.

The preparation of separate solutions of the isomeric glyceryl dinitrates was accomplished satisfactorily. About 70 % of each nitrate was obtained by extracting the silica gel collected from R_F 0.36 and R_F 0.47 after development in solvent 204. Rechromatography showed that the faster moving compound was uncontaminated (Table III). The slower dinitrate contained only 7 % of the R_F 0.47 isomer and was satisfactory for use in the hydrolysis experiments.

Hydrolysis of the faster migrating glyceryl dinitrate produced a single glyceryl mononitrate. This point was evident from both the radioscans and the autoradiograms of thin-layer plates developed in solvent 402 from the hydrolysate (Fig. 1).

The slower migrating glyceryl dinitrate yielded two glyceryl mononitrates upon

TABLE III

IDENTIFICATION OF GLYCERYL MONO- AND DINITRATES

Sample	*Medium*	*Time*	*No. of mono-nitrates produced***	*% Composition of hydrolysate**				
				Glycerol	*Glyceryl mono-nitrates*	*Glyceryl-1,2-di-nitrate*	*Glyceryl-1,3-di-nitrate*	*Glyceryl trinitrate*
Glyceryl dinitrate, R_F 0.36*	4 *N* HCl	0 h				93	7	
Glyceryl dinitrate, R_F 0.36*	4 *N* HCl	24 h	2	7.0	42.6	50.4		
Glyceryl dinitrate, R_F 0.47*	4 *N* HCl	0 h					100	
Glyceryl dinitrate, R_F 0.47*	4 *N* HCl	24 h	1	4.1	35.6		60.3	
Glyceryl trinitrate	Water	6 h						100
Glyceryl trinitrate	4 *N* HCl	6 h		0.5	1.6	17.7	8.1	72
Glyceryl trinitrate	4 *N* HCl	24 h	2		18.2	32.7	16.4	32.7
Glyceryl trinitrate	4 *N* HCl	48 h		8.5	32.7	31.9	15.5	11.5
Glyceryl trinitrate	4 *N* NaOH	15 min		98.4	1.6			

* Solvent 204.

** Determined with solvents 201 and 402.

acid hydrolysis. The resolution of these mononitrates in solvent 402 was visible on both the radioscan and the autoradiogram (Fig. 2). Although the radioscan suggests incomplete resolution of the isomeric mononitrates, these compounds were separated by a clear space of approximately 0.03 R_F units on the autoradiogram.

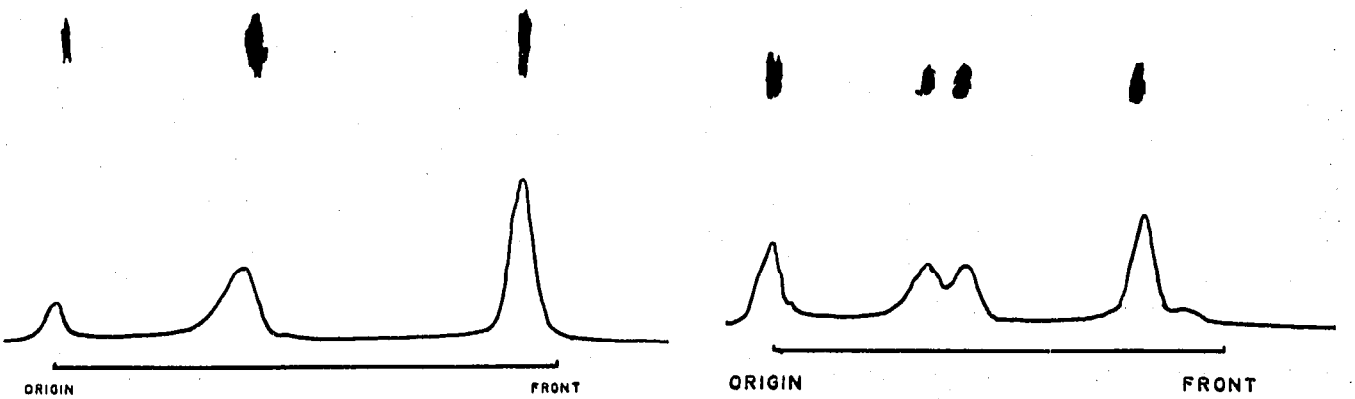


Fig. 1. Autoradiogram and radioscan of thin-layer chromatogram run in solvent 402 on acid hydrolysate of higher glyceryl dinitrate (R_F 0.47, solvent 204). Both detection systems show the presence of a single mononitrate (glyceryl-1-nitrate) at R_F 0.37. Also evident are glycerol at R_F 0.0 and glyceryl-1,3-dinitrate at R_F 0.94.

Fig. 2. Autoradiogram and radioscan of thin-layer chromatogram run in solvent 402 on acid hydrolysate of lower glyceryl dinitrate (R_F 0.36, solvent 204). Both detection systems clearly show the presence of two mononitrates in about equal amounts at R_F 0.35 and R_F 0.43; these are glyceryl-1-nitrate and glyceryl-2-nitrate, respectively. Glycerol (R_F 0.0) and glyceryl-1,2-dinitrate (R_F 0.83) are also evident.

The identification of glyceryl nitrate isomers is summarized in Table III. The slower migrating dinitrate (R_F 0.36 in solvent 204) was identified as glyceryl-1,2-dinitrate because it yielded two mononitrates upon acid hydrolysis. The faster migrating compound yielded only one mononitrate and was assigned the 1,3-dinitrate structure. These assignments were supported by the observation that the three acid hydrolyses of glyceryl trinitrate produced twice as much of the slower migrating glyceryl (1,2-) dinitrate than of the faster glyceryl (1,3-) dinitrate. Twice as much 1,2-dinitrate was expected because there are two primary nitrato groups and one secondary nitrato group in glyceryl trinitrate. Table III also illustrates the stability of glyceryl trinitrate in water, its rapid hydrolysis in alkaline solution, and the moderate rate of hydrolysis in 4 *N* HCl. The acid hydrolysis of the two dinitrates proceeded at comparable rates.

Table IV presents the R_F values obtained in a number of solvents for glycerol and for all of its nitrates.

DISCUSSION

The effort to identify the isomeric glyceryl mononitrates and dinitrates by thin-layer chromatography was successful. Since glyceryl-1,3-dinitrate can yield only glyceryl-1-nitrate on a partial hydrolysis, it became evident from the data that the faster moving dinitrate is the 1,3-dinitrate and the slower moving mononitrate is the 1-nitrate. The faster moving mononitrate is the 2-nitrate which can be obtained from the 1,2-dinitrate, but not from the 1,3-dinitrate. This finding does not support

TABLE IV

RESOLUTION OF GLYCEROL AND ITS NITRATES BY THIN-LAYER CHROMATOGRAPHY

Solvent	*R_F value*					
	Glycerol	*Mononitrates*		*Dinitrates*		*Trinitrate*
		1-	*2-*	*1,2-*	*1,3-*	
105	0.22	0.59*		0.77*		0.77
106	0.48	0.73*		0.82*		0.87
201	0.00	0.17	0.20	0.65	0.76	0.76
203	0.00	0.21*			0.37*	0.61
204	0.00	0.10*		0.36	0.47	0.63
401	0.09	0.67	0.69	0.86	0.89	>0.9
402	0.00	0.37	0.45	0.84	0.94	>0.9

* Isomers not resolved.

NEEDLEMAN AND HUNTER[5] who indicated that glyceryl-1,2-dinitrate migrates faster than the 1,3-dinitrate in benzene–ethyl acetate–acetic acid (80:20:5) which is called solvent 204 in the present report.

The hydrolysis of glyceryl trinitrate at 37° in 4 *N* hydrochloric acid removed each nitrato group at comparable rates. The ratio of the two dinitrates formed is a direct consequence of the availability for hydrolysis of two primary nitrates to each secondary nitrate in glyceryl trinitrate. Similarly, the two mononitrates were formed in equal amounts by the hydrolysis of glyceryl-1,2-dinitrate.

Ether extraction of an aqueous solution of mixed mono- and dinitrates removed 97 % of the dinitrates in four extractions and the remainder in the next four extractions. Only 69 % of the mononitrates was removed in all eight of these extractions. This low efficiency of mononitrate extraction serves as a precaution against the use of ether for the extraction of glyceryl nitrates from biological systems for assay purposes.

The methodology described above was applied successfully to the identification and assay of urinary drug metabolites excreted after administering radioactively labeled glyceryl trinitrate to rats[6].

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REFERENCES

1 W. WILL, *Ber.*, 41 (1908) 1107.
2 I. DUNSTAN, J. V. GRIFFITHS AND S. A. HARVEY, *J. Chem. Soc.*, (1965) 1319, 1325.
3 J. W. LAWRIE, *Glycerol and the Glycols*, Chemical Catalog Co., New York, 1928, p. 318.
4 A. OSOL AND G. E. FARRAR, *The Dispensatory of the United States*, 24th Ed., J. B. Lippincott, Co., Philadelphia, 1950, p. 431.
5 P. NEEDLEMAN AND F. E. HUNTER, JR., *Mol. Pharmacol.*, 1 (1965) 77.
6 F. J. DICARLO, M. C. CREW, L. J. HAYNES, M. D. MELGAR AND R. L. GALA, *Biochem. Pharmacol.*, in press.