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Separation and determination of the two components of glycerol formal by high-performance liquid chromatography*

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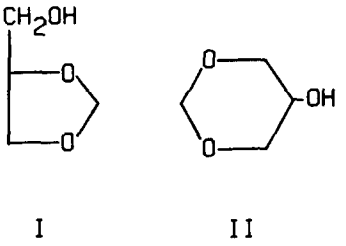
Abstract: The two isomeric components of glycerol formal, 1,3-dioxan-5-ol and 1,3-dioxolane-4-methanol, are marginally separated (R_s = 1.0) by polar-bonded-phase high-performance liquid chromatography (HPLC) on a cyanopropyl column with acetone–hexane as the eluent. Esterification of these components with 3,5-dinitrobenzoyl chloride produces derivatives which are, however, completely resolved (R_s > 2) by normal-phase HPLC on silica; derivatization has the added advantage of introducing an ultraviolet-absorbing chromophore into each component. Preparative scale chromatography is used to isolate each of the derivatives, which are characterized by their UV, NMR and mass spectral properties. These esters are used as reference standards for an analytical method based on derivatization and normal-phase chromatography. In this way a sample of glycerol formal is calibrated for use as a standard in the direct determination of the two components by polar-bonded-phase HPLC.

Keywords: *3,5-Dinitrobenzoyl chloride; 1,3-dioxan-5-ol; 1,3-dioxolane-4-methanol; glycerol formal; high-performance liquid chromatography; pre-column derivatization.*

Introduction

Glycerol formal is a mixture of two cyclic acetal isomers formed by the condensation of glycerin and formaldehyde [1]. The structures of the two components, 1,3-dioxolane-4-methanol (I) and 1,3-dioxan-5-ol (II), are shown in Fig. 1. Quantitative proton magnetic resonance has been used to demonstrate that the isomeric composition is approximately



I II

Figure 1
Structures of the two isomers of glycerol formal. I: 1,3-dioxolane-4-methanol; II: 1,3-dioxan-5-ol.

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40% dioxolane and 60% dioxane [2]. Since glycerol formal is a non-volatile, slightly viscous, polar organic solvent, it is used as a vehicle in a number of veterinary injections. In addition, one of the components, 1,3-dioxan-5-ol, is of interest because its molecular geometry suggests the possibility of an intramolecular, bifurcated hydrogen bond [3, 4].

Glycerol formal is usually determined by packed-column gas chromatography, but because the two isomers are not well separated, only total concentrations of glycerol formal can be measured by this technique [5]. However, capillary gas chromatography has been used to resolve and determine the two components in both animal plasma and tissue [5, 6]. The present report describes a method based on high-performance liquid chromatography (HPLC) that enables the two components of glycerol formal to be resolved and determined.

As a means of isolating the two components, glycerol formal can be converted to a mixture of its benzoate esters [1]. These esters can then be separated by fractional crystallization and subsequent hydrolysis to the free alcohols, although difficulty with this separation scheme has been reported [3]. This derivatization approach has also been used in the present work. Conversion of glycerol formal to its 3,5-dinitrobenzoate esters is a means of enhancing separation by HPLC so that the two components may then be isolated by preparative chromatography. In this way pure samples of the esters of the two components are obtained for use as reference standards in the HPLC analysis of glycerol formal with pre-column derivatization.

Experimental

Reagents

Several lots of glycerol formal were obtained from two manufacturers (Diamond Shamrock, Cleveland, OH, USA, and Chemie Linz AG, Austria). Reagent grade pyridine (J.T. Baker, Phillipsburg, NJ, USA) and 3,5-dinitrobenzoyl chloride (Aldrich, Milwaukee, WI, USA) were used as received. All solvents for both chromatography and synthesis were HPLC grade. The chloroform was free from alcohol and the tetrahydrofuran (THF) contained no UV-absorbing stabilizer. Distilled water was further purified for chromatography by passing through an 'ultrapure' water system (Super-Q, Millipore, Bedford, MA, USA).

Instrumentation

A liquid chromatograph was assembled from the following components: a constant-flow pump (Model 740B, Spectra Physics, Santa Clara, CA, USA); a loop injection valve (Model CV-6-UHPa-N60, Valco, Houston, TX, USA) equipped with either a 10-μl loop for analytical purposes or a 1.0-ml loop for preparative chromatography; either a filter photometric detector (Model SP8300, Spectra Physics, Santa Clara, CA, USA) or a refractive index detector (Model R401, Waters Associates, Milford, MA, USA); and an electronic integrator (Model HP3385A, Hewlett–Packard, Palo Alto, CA, USA). The chromatographic column was completely enclosed in a water jacket maintained at constant temperature with a circulating bath (Model FS, Haake Instruments, Saddle Brooke, NJ, USA).

Ultraviolet spectra (Model HP8450A, Hewlett–Packard, Palo Alto, CA, USA) were recorded in 1-cm cells using acetonitrile both as solvent and reference. Electron impact ionization mass spectra (Model 9000, LKB, Gaithersburg, MD, USA) were obtained using a potential of 70 eV. Nuclear magnetic resonance spectra were recorded for

solutions (5 mg/ml) in deuterated chloroform with a 200-MHz Fourier transform spectrometer (Model XL 200, Varian, Palo Alto, CA, USA) using a 1-s acquisition time, a flip angle of 45°, and tetramethylsilane (TMS) as reference. Thermal analysis was by differential scanning calorimetry (Model DSC-1B, Perkin–Elmer, Norwalk, CT, USA).

HPLC conditions

For glycerol formal: a 6-μm Zorbax CN column 250 × 4.6 mm i.d. (DuPont, Wilmington, DE, USA) maintained at 25°C, was used with acetone–hexane (15:85 v/v) as the eluent and with refractive index detection.

For analysis of the dinitrobenzoates of glycerol formal: a 5-μm Spherisorb Silica S5W column 250 × 4.6 mm i.d. (Phase Separations, Norwalk, CT, USA) maintained at 33°C, was used with THF–hexane (25:75 v/v) as the eluent and with photometric detection at 254 nm.

For isolation of the individual dinitrobenzoates: a 10-μm Partisil 10-M9 column 250 × 9.4 mm i.d. (Whatman, Clifton, NJ, USA) at ambient temperature was used with THF–hexane (30:70 v/v) as the eluent and with photometric detection at 312 nm. In general, samples for injection were dissolved in the chromatographic eluent.

Preparation of glycerol formal 3,5-dinitrobenzoates

Pyridine (20 ml) was added to a solution of 3,5-dinitrobenzoyl chloride (33 g) in chloroform (200 ml) at ambient temperature. A precipitate formed almost immediately. Addition of glycerol formal (10 ml) resulted in dissolution of the precipitate in *ca* 1 min. After the resulting solution had cooled to ambient temperature, it was washed successively with 400-ml portions of 0.5 M HCl, water, a saturated solution of sodium bicarbonate, and additional water, respectively. The chloroform phase was dried by passage through several layers of filter paper; its volume was then reduced and crystallization induced by addition of a hydrocarbon solvent (*iso*-octane). After collection of the solid product mixture, a saturated solution was prepared in THF–hexane (30:70 v/v), and the individual components were isolated by repetitive preparative scale HPLC as described above. The pooled, collected fractions for each component were evaporated to dryness, and the products were recrystallized using THF and *iso*-octane.

Analytical derivatization procedure

To a 2.00-ml solution of glycerol formal (0.1–1.0 mg/ml) in alcohol-free chloroform was added 2.00 ml of a 4% m/v solution of 3,5-dinitrobenzoyl chloride in the same solvent and 0.10 ml of pyridine. After 0.5 h the reaction mixture was washed successively with 10-ml portions of 1.0 M NaOH, water, 2.0 M HCl and additional water and injected into the chromatograph. The analytical procedure was applied to solutions of the two reference standards of glycerol formal dinitrobenzoates in alcohol-free chloroform.

Results and Discussion

HPLC

Initial studies by reversed-phase chromatography with an octadecylsilyl column and by normal-phase chromatography with a silica column showed no resolution of the two

glycerol formal components. However, the use of polar-bonded-phase chromatography with a cyanopropyl column resulted in some resolution. In the reversed-phase mode with methanol–water as the eluent the resolution was minimal, but in the normal-phase mode with acetone–hexane as the eluent the chromatography was somewhat improved. A typical chromatogram corresponding to this latter system is shown in Fig. 2. The resolution between the two peaks, calculated as the difference in retention times divided by the sum of the peak widths at half height, is approximately 1.0. For symmetrical peaks of about the same size, this resolution corresponds to an area overlap of approximately 2% [7]. Furthermore, if peak heights are used for quantitation, there is essentially no interference between the two peaks. However, this degree of resolution is insufficient for isolation of the two individual isomers by preparative chromatography even on a small scale.

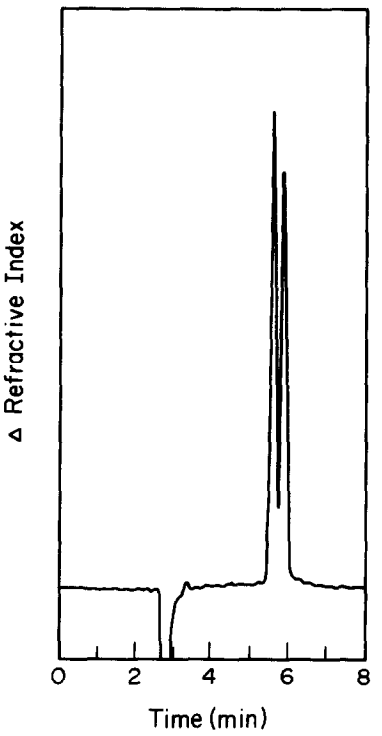

Figure 2
HPLC separation of the components of glycerol formal on a cyanopropyl column. The dioxane component elutes at 5.6 min and the dioxolane at 5.9 min. Flow rate, 2 ml/min; attenuation, 32×; total injected, 0.2 mg; other conditions as in text.

Complete separation of the 3,5-dinitrobenzoate esters of glycerol formal on both analytical and preparative scales was achieved on a silica column with THF–hexane as the eluent, as illustrated in Fig. 3. In this chromatogram the two later-eluting peaks correspond to the dinitrobenzoates; the early-eluting peaks are attributable to solvent and excess reagents used in the esterification procedure. With a column of larger diameter and a minor modification to the eluent, this normal-phase chromatographic system was used to isolate the two components, permitting approximately 0.5 mg of each component to be collected from a single chromatographic run.

Preparation and isolation of glycerol formal 3,5-dinitrobenzoates

On a preparative scale, the reaction of glycerol formal with a slight molar excess of 3,5-dinitrobenzoyl chloride occurred rapidly at ambient temperature in a chloroform

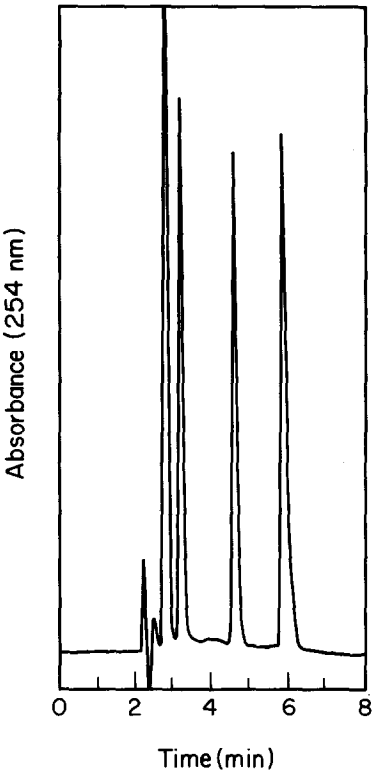

Figure 3
HPLC separation of the 3,5-dinitrobenzoates of glycerol formal on a silica column. The dioxolane ester elutes at 4.7 min and the dioxane at 5.9 min. Flow rate, 1.5 ml/min; attenuation, 0.16 a.u.f.s.; total injected, 0.004 mg; other conditions as in text.

solution containing an equivalent amount of pyridine. Liquid–liquid partitions with aqueous acid and base effectively removed the pyridine and excess reagent, and the mixture of reaction products was further purified by recrystallization. Approximately 20 mg of each of the two products were obtained by repetitive preparative HPLC.

The choice of 3,5-dinitrobenzoyl chloride as a derivatization reagent, rather than unsubstituted benzoyl chloride, was made for a number of reasons. First, the reaction proceeded faster with the nitro-substituted reagent; this was particularly significant on the analytical scale. Second, the higher melting points of the dinitrobenzoates facilitated crystallization of the products. Finally, the nitro-substituents on the aromatic ring enhance the UV absorption of the chromophore [8].

Characterization of glycerol formal 3,5-dinitrobenzoates

To confirm the identity of the dinitrobenzoate esters of glycerol formal, their mass spectra were obtained as summarized in Table 1. For each component a small parent peak was seen in addition to major peaks for each half of the molecule. No intense peaks were seen which could not be attributed to potential fragments of the glycerol formal dinitrobenzoate molecule. However, the fragmentation patterns gave no indication as to which component was the dioxolane and which the dioxane.

Since the UV absorption spectra of equal concentrations of the two dinitrobenzoates in acetonitrile solution were found (Fig. 4) to be superimposable at all wavelengths above 235 nm, the HPLC peak areas measured for glycerol formal by UV detection after derivatization were used to calculate the isomer ratio. The chromatogram in Fig. 3 indicates that this ratio, in terms of earlier- to later-eluting peaks, is 2:3; this is in

Table 1
Mass spectra of glycerol formal dinitrobenzoate isomers

Mass number	Relative intensity*		Fragment†
	Component 1	Component 2	
298	2	1	P^+
297	8	8	P^+-H
268	6	5	P^+-NO
238	6	95	$P^+-2(NO)$
195	84	100	$(NO_2)_2C_6H_3CO^+$
179	8	10	$(NO_2)_2C_6H_3C^+$
149	36	72	$NO_2C_6H_3CO^+$
103	16	22	GF^+-H
86	71	82	GF^+-H_2O
75	92	96	$C_6H_3^+$
74	35	22	$C_6H_2^+$
73	100	17	C_6H^+
46	100	29	NO_2^+

* Component 1 elutes first in the normal-phase HPLC system.
† P = parent ion; GF = glycerol formal ($C_6H_8O_3$).

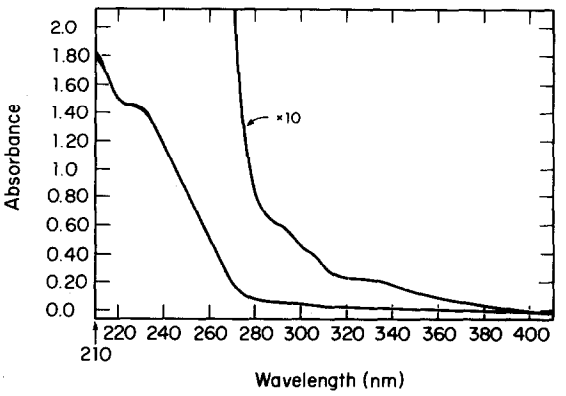

Figure 4
Ultraviolet absorption spectra of the two glycerol formal dinitrobenzoates in acetonitrile. Concentration of each component is 0.022 mg/ml; '×10' indicates electronic amplification.

agreement with the anticipated isomer ratio and indicates that the dinitrobenzoate of the dioxolane component is eluted first.

This elution order was confirmed by 200-MHz proton magnetic resonance (PMR) spectrometry. Even at this high field, most of the signals for both components were not well resolved. However, the aliphatic signals furthest downfield from TMS were well defined; these can be assigned to protons of the methylene group located between the two ether oxygens. For the later-eluting component, an AB-quartet pattern was seen with the two groups centred at chemical shift values of 4.88 and 5.08 ppm and with a coupling constant of 6 Hz. The corresponding signals for the earlier-eluting component were somewhat different: two singlets of equal intensity were seen with chemical shifts of 4.98 and 5.17 ppm. These two signals could represent either the inner lines of an AB-quartet, whose outer lines are not sufficiently intense to be detected, or they could

actually correspond to singlets, reflecting negligible geminal coupling. Based on the PMR spectrum of glycerol formal itself [2], the conventional AB-quartet observed for the later-eluting dinitrobenzoate ester can be assigned to the dioxane structure and the two singlets of the earlier-eluting ester to the dioxolane structure.

In the PMR spectra of both dinitrobenzoate esters of glycerol formal, the diether methylene signals are well defined; this suggests that in the case of both the dioxane and dioxolane components only one conformation is present. For steric reasons the aromatic group is likely to be in the equatorial position in both components.

Examination of the isolated dinitrobenzoates by normal-phase HPLC with UV detection showed no evidence of cross-contamination, nor of extraneous impurities with UV absorption at 254 nm. Their purity was further demonstrated by differential scanning calorimetry, for which the properties measured are listed in Table 2. Whereas the dioxane component was very pure, the dioxolane component was somewhat less pure on a molar basis. Since these materials were isolated by preparative HPLC, the only impurities likely to have been present are the low-molecular-weight compounds used in the chromatographic eluent and the solvent used for recrystallization. Thus, the purity of the dioxolane component was probably somewhat greater than 99% m/m, and a value of 100% was assumed for calculation purposes.

Table 2
Differential scanning calorimetry of glycerol formal dinitrobenzoate components

	Dioxolane	Dioxane
Melting point	75°C	172°C
Heat of fusion	6.2 kcal/mol	9.1 kcal/mol
Purity (mole %)	98.7%	99.9%

Properties of the analytical derivatization procedure

Figure 5 shows the time course of formation of glycerol formal dinitrobenzoate on an analytical scale monitored by normal-phase HPLC. Although the dioxane component reacts more slowly than the dioxolane component, both reactions are complete after 30 min at ambient temperature, and no degradation of either product can be detected in the reaction medium for at least another 30 min.

The determination by derivatization of the two components in the concentration range 0.1–1.0 mg/ml of glycerol formal is illustrated in Fig. 6. In these analyses the two isolated glycerol formal dinitrobenzoates were used as reference standards. The response for each component is linear over the concentration range examined; moreover, the sum of each linear response curve falls on the diagonal, confirming that the derivatization reactions proceed quantitatively for both components.

The results for replicate determinations performed on a single sample of glycerol formal on three separate days are summarized in Table 3. The overall reproducibility (RSD) of the procedure was 3%, and the within-run and between-run measures of precision were essentially equal.

Direct analysis of glycerol formal

The analytical results using dinitrobenzoate derivatization on two lots of glycerol formal with significantly different isomer ratios were compared to the results of direct

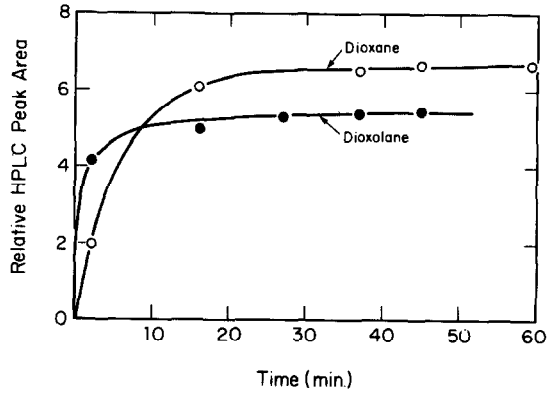

Figure 5
Time course of glycerol formal derivatization at 25°C. Reaction conditions as in text; the initial concentration of glycerol formal was 0.4 mg/ml in chloroform.

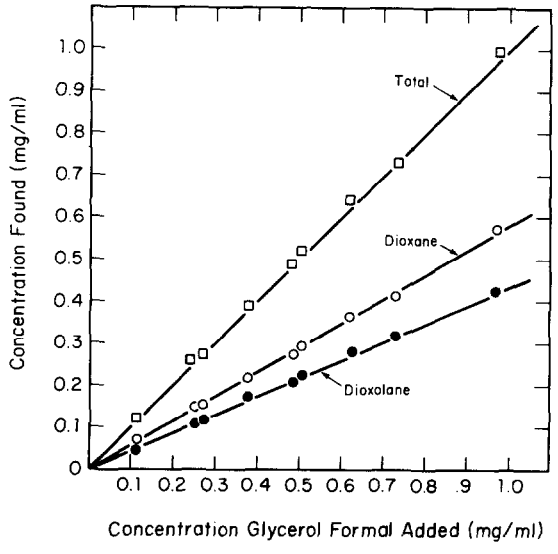

Figure 6
Determination of glycerol formal components by derivatization with 3,5-dinitrobenzoyl chloride. The 'total' line represents the sum of the concentrations observed for each component.

chromatography on the polar-bonded-phase system with refractive index detection. Although the derivatization procedure gave absolute concentrations for each of the two components, direct chromatography could only measure relative peak areas, because neither the refractive index nor the elution order of the two components was known. The results are summarized in Table 4.

The derivatization procedure demonstrated that lot No. 5 contained more of the dioxane component than did lot No. 1. Furthermore, the relative area of the first peak eluting in the direct chromatographic system was higher for lot No. 5 than it was for lot No. 1; this result indicated that the dioxane component of glycerol formal elutes first from the polar bonded-phase column used in the normal-phase mode. The correspond-

Table 3
Component assay of glycerol formal by the dinitrobenzoate method*

Assay†	Dioxolane	Dioxane	Mean dioxolane	Mean dioxane	Total
1	43.4	58.8	43.0	57.0	100.0
	42.9	56.9			
	42.8	56.4			
	43.0	56.1			
2	44.5	59.2	44.6	59.0	103.6
	43.9	58.6			
	44.3	58.2			
	44.7	59.1			
	45.8	59.8			
3	42.3	55.5	42.1	55.0	97.1
	42.1	55.0			
	41.8	54.6			
Overall	43.5 ± 1.2‡	57.4 ± 1.8‡			100.9 ± 3.0‡

* The data were obtained on Lot No. 1.
† The groups of replicate assay data were each obtained on different days.
‡ Standard deviation.

Table 4
Comparison of two HPLC methods for determining the components of glycerol formal

	Derivatization method*		Direct method†	
Lot No.	Dioxane	Dioxolane	First peak	Second peak
1	57.4	43.5	57.2	42.8
5	60.2	38.1	62.1	37.9

* Results expressed as per cent by weight.
† Results expressed as per cent of total peak area.

ing situation is true for the dioxolane component. In fact, in all cases the weight-per cent values obtained by the derivatization procedure was very close to the per cent-of-total-peak-area values obtained by direct chromatography with refractive index detection. This demonstrates that by coincidence the refractive indices of the two components of glycerol formal are essentially equal.

After the elution order of the two components had been established, one lot of glycerol formal which had been repeatedly analysed by the derivatization procedure (cf. Table 3) was used as a reference standard for the direct analysis of various other glycerol formal samples by polar-bonded-phase chromatography. The results (Table 5) indicate that there is a small difference in the isomer ratios produced by the two different manufacturers. This difference is fortuitous because it facilitated the determination of the elution order in the polar-bonded-phase chromatographic system. Without this difference, isolation of the two individual components of glycerol formal as the free alcohols would have been necessary. The analytical results also show a slight difference

Table 5
Direct component assay of various lots of glycerol formal

Lot No.	Manufacturer	Dioxane (%)	Dioxolane (%)	Total (%)
2	A	56.7	41.9	98.6
3	A	57.1	40.4	97.5
4	A	60.4	41.8	100.7
5	B	61.8	38.4	100.2
6	B	61.7	38.4	100.1
7	B	61.4	41.9	103.3

in overall purity between samples from the two manufacturers, but this difference is probably within the variability of the analytical measurement.

Conclusions

Two HPLC methods for determining the components of glycerol formal have been presented. One is a direct procedure using polar-bonded-phase chromatography on a cyanopropyl column with refractive index detection. This method is fast and simple but the resolution of the two components is only marginally adequate and the limit of detection is not very low. The other method entails conversion of the components to their 3,5-dinitrobenzoate esters followed by normal-phase chromatography on silica with UV-photometric detection. Whereas this method is more complex than the direct method, it gives linear and reproducible results, with complete resolution of the two components, and the derivatization reaction is quantitative. The limit of detection is <0.01%, which is more than adequate for analyses of pharmaceutical preparations. Also, the electro-active nature of the dinitrobenzoyl group should make possible analyses at very low levels by HPLC with electrochemical detection. Primary standards for the derivatization method can be prepared as the dinitrobenzoates and isolated as pure crystalline materials by preparative HPLC. There is no need to isolate the underivatized components of glycerol formal, since the mixture of free alcohols can be standardized by the derivatization method as a reference material for analysis by the direct method.

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