CHROM. 5329

GAS CHROMATOGRAPHIC SEPARATION AND DETECTION OF PENTAERYTHRITOL NITRATES AND OTHER ORGANIC NITRATE ESTERS*

I. W. F. DAVIDSON, F. J. DICARLO AND E. I. SZABO
Department of Pharmacology, Bowman Gray School of Medicine, Wake Forest University, Winston Salem, N. C. 27103 (U.S.A.)
(Received February 22nd, 1971)

SUMMARY

A gas chromatographic method for the separation and quantitation of submicrogram amounts of pentaerythritol tetranitrate and other organic nitrate esters of common therapeutic use is presented. The procedure allows the quantitative simultaneous analysis of pentaerythritol tetranitrate, the trifluoroacetylated derivatives of its lesser nitrate esters and pentaerythritol on a single column. For rapid, routine analysis the flame ionization detection is employed with a sensitivity in the micro- to submicrogram range. With electron capture detection, the sensitivity is in the nano- to subnanogram range. This sensitivity is adequate for quantitating the low levels of pentaerythritol and pentaerythritol nitrate esters in biological fluids.

INTRODUCTION

Certain of the polynitrate esters of alkyl alcohols, for example pentaerythritol tetranitrate (PETN), erythritol tetranitrate, isosorbide dinitrate, mannitol hexanitrate, have wide use as vasodilators in the therapy of angina pectoris. Completely satisfactory methods are not available for identifying and determining these compounds together with their lesser nitrate esters which may occur in very small amounts as drug impurities or as metabolites in body fluids and tissues. Specific identification of these drugs can be accomplished most readily by thin-layer chromatography (TLC)[1,2]. Quantitation by spectrophotometric methods of individual compounds when other esters are known to be absent is well established[3–5], but estimation of individual nitrates in a mixture of esters after separation on TLC plates is time consuming and limited in precision and sensitivity[6–8]. For metabolic studies, TLC combined with radiometric scanning has been employed to determine biological metabolites after the administration of [^{14}C]PETN[9]. This method suffers from the disadvantage that radioactive drug is required for quantitation, and also it is insufficiently sensitive for determinations in individual samples of blood or plasma[10].

* This study was supported in part by USPHS grant RR00336.

Gas chromatography (GC) has been applied to organic nitrates but, with the exception of nitroglycerin[11,12], not to the commonly used pharmaceuticals, nor to the separation and detection of a series of lesser esters from a parent drug. Chromatography of a polynitrate and its lesser nitrate esters on one column presents unusual difficulties because of wide differences in polarity, solubility and thermal stability of these compounds.

In this paper we describe a GC method for the rapid separation and identification of several of the nitrate esters of therapeutic interest. In addition, as illustrated for PETN, the method can be used to determine simultaneously the individual lesser nitrate esters and their alkyl alcohol as trifluoroacetyl derivatives. The method for PETN provides a rapid, routine procedure for general analytical use with detection of micro- and submicrogram quantities with the flame ionization detector; and with the electron capture detector to determine nano- to subnanogram quantities, adequate sensitivity for application to the detection of these compounds in biological samples.

EXPERIMENTAL

Gas chromatography conditions

The chromatograph was a Varian Aerograph, model 2100-20 with a model 20 variable speed recorder (10 in.) and equipped with a hydrogen flame ionization detector (FID) and a tritium (250 mCi) electron capture detector (ECD). Varian U-shaped glass columns, 2 mm I.D. × 1/4 in. O.D. were used. The "SE-30 column" (67 in.) was packed with 100–120 mesh washed and trimethylchlorosilylated Chromosorb P which had been coated with 1% dimethylsilicone gum (GC-grade SE-30, Analabs, Inc., North Haven, Conn.). The "Dexsil column" (72 in.) was packed with 100–120 mesh washed and dimethylchlorosilylated Chromosorb W with a liquid phase of 1% carboranesiloxane (Dexsil 300 GC, Analabs, Inc.). Septa were cleaned by thoroughly washing with distilled water, acetone, and hexane (nanograde solvent) and finally heated at 250° for several days. Septa W-9 (Applied Science Lab., Inc., State College, Penn.) were used with the SE-30 column and septa 69-10 (Varian Aerograph) with the Dexsil column.

The injection port and detectors were maintained at 200° with nitrogen as the carrier gas. The columns were conditioned with trifluoroacetyl derivatives of the nitro compounds immediately before analytical use by repetitive injection of a composite solution of derivatives until a reproducible response was obtained.

For FID detection the gas flow was hydrogen 20 ml/min, air 300 ml/min, and the carrier gas 20 ml/min. The columns were programmed at a rate of 10°/min with an initial setting of 65°.

For ECD detection, the carrier gas flow was 75 ml/min. The columns were operated isothermally with temperature settings as noted.

Peak areas were determined with a compensating polar planimeter.

Preparation of sample derivatives

About 400 μg of sample or individual compounds previously dried were dissolved in 200 μl of chloroform or dichloromethane (nanograde solvents) in 3.5 ml reaction vials (Pierce Chemical Co., Rockford, Ill.). Then 200 μl of trifluoroacetic anhydride (TFA) were added and the mixture allowed to stand for 30–45 min with occasional

agitation. Derivatization proceeded rapidly in solution at room temperature and it was essentially complete in 30 min. The reaction mixture was evaporated to dryness at room temperature with a dry nitrogen stream and the residue redissolved in nanograde solvents: dichloromethane or carbon tetrachloride–ethyl acetate (7:1) for FID, or in ethyl acetate for ECD.

The reaction conditions for derivatization were adopted as the result of the following experiments. Pentaerythritol (PE) and individual pentaerythritol nitrate esters were derivatized as outlined. The reaction mixtures were chromatographed on SE-30 and Dexsil columns with FID at 5.0 min intervals for 60 min after addition of TFA. Constant single peak areas with disappearance of underivatized compound was observed after 20 min for all compounds. A similar result was obtained with a composite solution of compounds: PE 20 μg; PE mononitrate 32 μg; PE dinitrate 70 μg; PE trinitrate 100 μg; and PE tetranitrate 120 μg in 40 μl dichloromethane. After evaporation of the reaction mixture with a dry nitrogen stream at room temperature, re-solution with dichloromethane or ethyl acetate established that no loss of the derivatives occurred even after repeated evaporation. Solutions of the individual compounds were allowed to stand at room temperature in the reaction mixture with TFA and chromatographed at intervals over a period of 24 h. Single peaks were obtained up to 8 h but thereafter multiple peaks, evidence of decomposition, appeared. In pure solvent, the trifluoroacetyl derivatives were stable for at least one week stored at 10°.

Reference samples

PE, PE mono-, di-, tri- and tetranitrates were obtained from Warner-Lambert Research Institute, Morris Plains, N.J. The compounds were ascertained to give single spots with TLC[9] and single peaks with GC on SE-30 and Dexsil columns. Several of the compounds, PE mono-, di-, and tetranitrate were supplied with lactose as a diluent. To remove the lactose, these mixtures were extracted repeatedly with acetone (chromatoquality grade, Matheson, Coleman and Bell) or ethyl acetate (chromatoquality grade). Standard solutions were prepared in acetone (PE in acetone–water, 99:1) or ethyl acetate, at a concentration of 400 μg/ml. They were stable indefinitely at room temperature.

Mannitol hexanitrate (Atlas Chemical Co., Wilmington, Del.), erythritol tetranitrate (Burroughs Wellcome Co., Tuckahoe, N.Y.) and isosorbide dinitrate (Ives Laboratories, Inc., New York, N.Y.) were obtained diluted with lactose. The nitrates were extracted from the drug–lactose mixtures with dichloromethane and stock solutions of the nitrate drugs, 400 μg/ml, were prepared in dichloromethane.

RESULTS AND DISCUSSION

The chromatogram of Fig. 1a demonstrates that an adequate separation of a mixture of polynitrate drugs (erythritol tetranitrate, isosorbide dinitrate, pentaerythritol tetranitrate and mannitol hexanitrate) can be obtained for identification and quantitation purposes. The polynitrate drug mixture was directly injected onto an SE-30 column and temperature programmed at 10°/min. The procedure allows detection with quantitation of nanogram amounts of these compounds at the attenuation shown. Similar results were obtained with a different stationary liquid phase, Dexsil 300 GC, with identical chromatography conditions. Retention values for both

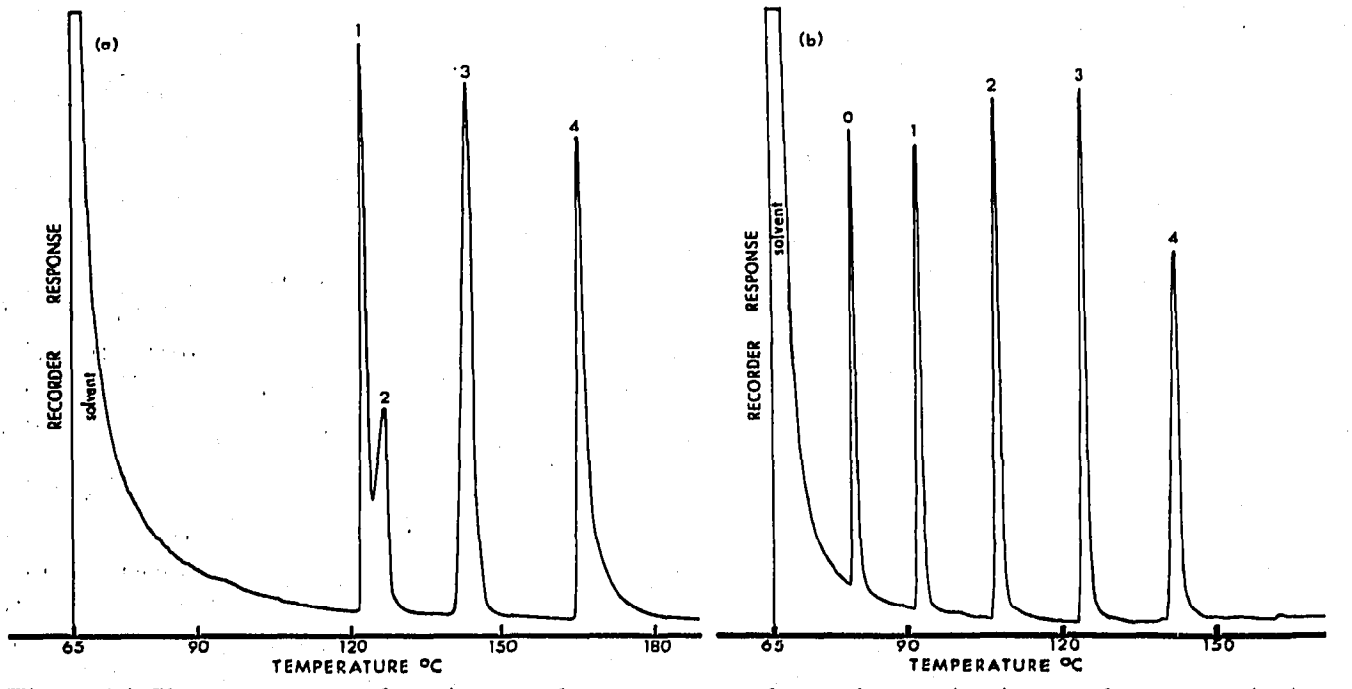

Fig. 1. (a) Chromatogram of a mixture of some commonly used organic nitrate pharmaceuticals on a 1% SE-30 column. 1 = isosorbide dinitrate, 1.6 μg; 2 = erythritol tetranitrate, 1.6 μg; 3 = pentaerythritol tetranitrate, 4 μg; 4 = mannitol hexanitrate, 2 μg in an injection volume of 1.0 μl dichloromethane. Detector: flame ionization at attenuation 1×10^{-10} A. (b) Chromatogram of trifluoroacetylated pentaerythritol nitrates on a 1% SE-30 column: 0 = PE, 0.28 μg; 1 = PE mononitrate, 0.52 μg; 2 = PE dinitrate, 1.40 μg; 3 = PE trinitrate, 2.15 μg; 4 = PETN, 2.75 μg in an injection volume of 1.1 μl dichloromethane. Detector: flame ionization at attenuation 1×10^{-10} A.

TABLE I

RETENTION AND RESPONSE VALUES OF NITRATE ESTERS WITH FLAME IONIZATION DETECTION

	Retention		*Response*[a] ($mm^2/\mu g$)
	Time (min)	*Temperature (°C)*	
SE-30 column[b]			
PE	1.6	83	1305
PE mononitrate	2.8	96	855
PE dinitrate	4.4	111	395
PE trinitrate	6.1	128	275
PE tetranitrate	7.8	146	185
Isosorbide dinitrate	6.0	126	
Erythritol tetranitrate	6.3	130	
Mannitol hexanitrate	10.1	170	
Dexsil column[b]			
PE	2.5	90	1265
PE mononitrate	4.0	106	835
PE dinitrate	5.7	124	395
PE trinitrate	7.6	144	235
PE tetranitrate	9.6	165	195
Isosorbide dinitrate	7.8	146	
Erythritol tetranitrate	8.2	150	
Mannitol hexanitrate	11.6	185	

[a] Calculated as underivatized compound.
[b] Programmed at 10°/min, initial temperature 65°, attenuation 1×10^{-10} A.

columns are given in Table I. Several commercial preparations containing nitrate esters alone or in combination with other drugs were chromatographed and no interferences were noted.

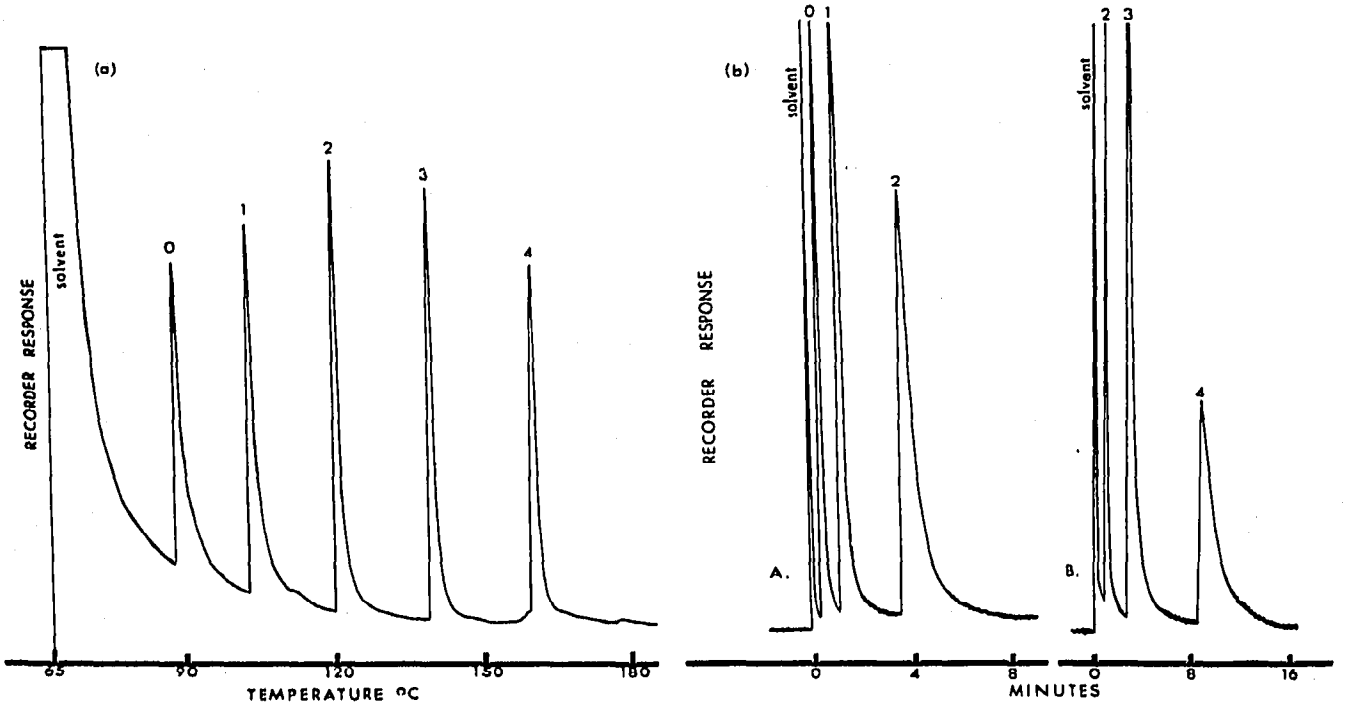

Fig. 2. (a) Chromatogram of trifluoroacetylated pentaerythritol nitrates on a 1% Dexsil column: 0 = PE, 0.40 μg; 1 = PE mononitrate, 0.64 μg; 2 = PE dinitrate, 1.40 μg; 3 = PE trinitrate, 2.00 μg; 4 = PETN, 2.40 μg in an injection volume of 0.8 μl carbon tetrachloride–ethyl acetate (7:1). Detector: flame ionization at attenuation 1×10^{-10} A. (b) Trifluoroacetylated pentaerythritol nitrates chromatographed on a 1% Dexsil column at isothermal conditions with electron capture detection. (A) Temperature 100°. 0 = PE, 8.3 ng; 1 = PE mononitrate, 15.6 ng; 2 = PE dinitrate, 42.5 ng in an injection volume of 0.5 μl ethyl acetate. Attenuation 8×10^{-9} A. (B) temperature 125°. 2 = PE dinitrate, 42.5 ng; 3 = PE trinitrate, 51.0 ng; 4 = PETN, 83.4 ng in an injection volume of 0.5 μl ethyl acetate. Attenuation 16×10^{-9} A.

Figs. 1b and 2a show the excellent separation of the drug PETN, the trifluoroacetyl derivatives of the lesser pentaerythritol nitrates and of PE on SE-30 and Dexsil columns respectively when a mixture of these compounds was derivatized and injected onto the columns. The two columns were similar in their properties with respect to resolution and response (peak area/μg), although the SE-30 column provided slightly narrower, more symmetrical peaks with less tailing.

A principal advantage of the Dexsil column is the greater thermal stability of this liquid phase as compared to SE-30. Column bleed from the SE-30 column, although low, was significantly greater than that from the Dexsil column. The bleed resulted in gradual shortening of retention times and limited the usefulness of the SE-30 column to 2–3 weeks. The extremely low bleed of the Dexsil column allowed this column to be used for 2–3 months, and also the low bleed was of advantage for use with ECD because of the sensitivity of this detector to contamination.

Table I lists retention and response values for the two columns. PETN, which having no free hydroxyl group is not derivatized with TFA, eluted from the Dexsil column at 165°, 20° higher than from the SE-30 column. The Dexsil column provided

more optimal separation (Table I) consistent with thermal stability. PETN (m.p. 140°) decomposed on the columns at temperatures of 175° or above. Experiments with longer columns of SE-30 and Dexsil or an increase in percentage of liquid phase resulted in increased elution temperatures and decomposition of PE trinitrate and PETN, while shorter columns demonstrated reduced resolution. Other liquid phases, of greater polarity, provided greater separation of PE, PE mononitrate, and PE dinitrate but decomposition of the higher nitrates occurred. Similarly, trimethylsilyl derivatives were more susceptible to temperature decomposition than trifluoroacetyl derivatives.

The chromatograms of Figs. 1b and 2a were obtained with an electrometer setting of 1×10^{-10} A with the flame ionization detection. This intermediate sensitivity setting was convenient for routine analysis. At higher attenuation, before increase in solvent peak width proved a limitation, the limits of detection for quantitation approximated 5 ng for PE and 50 ng for PETN with intermediate values for the other compounds. Fig. 3 shows the linearity of the response over a wide range of absolute amounts of PE and PETN.

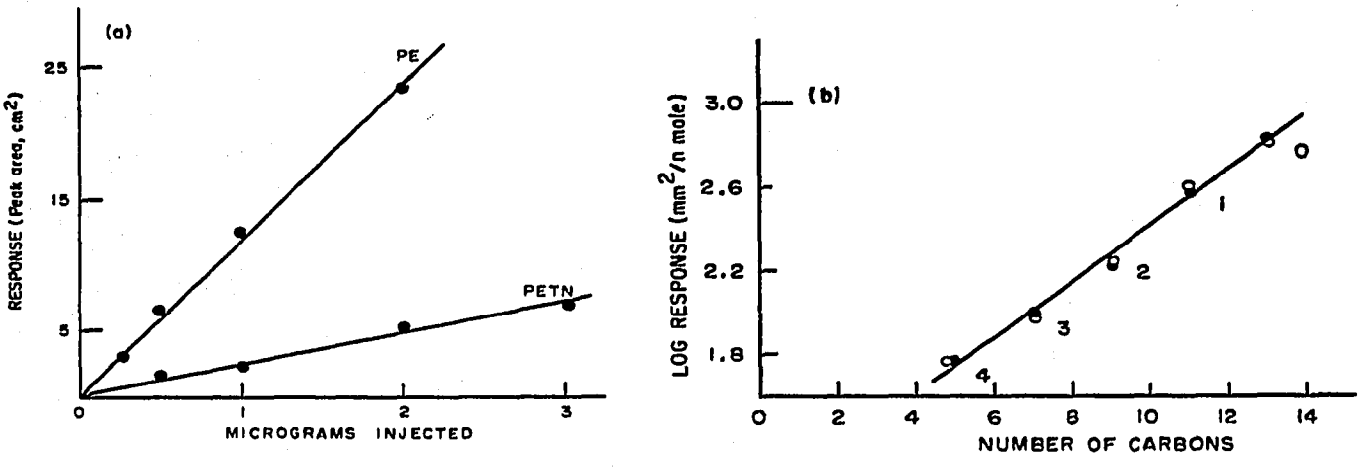

Fig. 3. (a) Calibration curves for PE and PETN injected onto a Dexsil column after treatment with trifluoroacetic anhydride as given in text. Attenuation 1×10^{-10} A. (b) Relationship of a number of carbon atoms of trifluoroacetylated PE (0), PE mononitrate (1), PE dinitrate (2), PE trinitrate (3) and PETN (4) with the peak areas obtained per nmole injected on an SE-30 column (●) and a Dexsil column (○). Attenuation 1×10^{-10} A.

As the FID response normally increases proportionally to the number of carbon atoms in the molecule, the trifluoroacetyl derivative of PE produced the greater response. A non-linear decreasing order of response from PE to PETN for equivalent mole amounts of the trifluoroacetylated compounds (Table I) results from the presence in the derivative compounds of varied proportional numbers of fluoro and nitro groups (and in PETN of nitro groups) both of which decreased the flame ionization current. However, a linear relationship for response with carbon atom number was obtained when the logarithm of the response was plotted against carbon number (Fig. 3b). This empirical function can be used to obtain or verify correction factors for the different detector responses to the compounds. Only two highly pure compounds are required, PE and PETN, both of which are readily obtainable, whereas the inter-

mediate compounds are difficult to obtain in pure form. The function was independent of the nature of the liquid phases of the two columns (Fig. 3b).

An increase in sensitivity of determination of pentaerythritol nitrates to the nano- to subnanogram range was possible with electron capture detection. Fig. 2b shows the chromatography of pentaerythritol and pentaerythritol nitrates isothermally on the Dexsil column. Similar separation was obtained with the SE-30 column. In practice, it was found that these five compounds could not be chromatographed at any single temperature without distortion of the peaks and extremely prolonged elution times for PE dinitrate, PE trinitrate and PETN. By chromatographing at two selected temperatures, as shown in Fig. 2b (A and B), well resolved, sharp symmetrical peaks with short retention times were obtained. Because of the different polarities of the two columns, the optimal temperatures for the Dexsil column (100° and 125°) were substantially higher than those for SE-30, 75° and 110°. At the lower column temperatures three compounds, PE, PE mononitrate and PE dinitrate, were obtained, at the higher temperatures PE dinitrate, PE trinitrate and PETN, providing an overlap for PE dinitrate. The procedure adopted was to chromatograph the sample isothermally at the lower temperature, then after the appearance of the PE dinitrate peak, the column was temperature programmed to 200° at 10°/min to remove the higher nitrate esters. Isothermal conditions were then reestablished at the higher temperature and the sample again injected. At the higher isothermal temperature all five compounds appear; but the retention times for PE and PE mononitrate are very short and these compounds eluted virtually with the solvent peak.

TABLE II

RETENTION AND RESPONSE VALUES OF PENTAERYTHRITOL NITRATES WITH ELECTRON CAPTURE DETECTION

	Retention time (min)	*Response (mm^2/ng)*[a]
SE-30 column		
Isothermal 75°		
PE	0.47	66.0
PE mononitrate	0.71	54.0
PE dinitrate	4.71	28.5
Isothermal 110°		
PE	0.16	—
PE mononitrate	0.24	—
PE dinitrate	0.63	18.0
PE trinitrate	0.94	41.5
PE tetranitrate	4.55	24.5
Dexsil column		
Isothermal 100°		
PE	0.47	26.3
PE mononitrate	1.24	24.3
PE dinitrate	3.76	13.0
Isothermal 125°		
PE	0.12	—
PE mononitrate	0.35	—
PE dinitrate	0.94	6.5
PE trinitrate	2.82	15.5
PE tetranitrate	8.82	8.5

[a] Calculated as underivatized compound; attenuation 16×10^{-9} A.

The retention times and detector response (peak area/ng) for the PE compounds on the Dexsil and SE-30 columns are given in Table II. Since the response of the EC detector decreased with an increase in temperature, the sensitivity at the two isothermal temperatures was not comparable; and the response for PE dinitrate, which appears at both temperatures, was decreased one-half by the higher temperature (Table II). Similarly Table II shows a greater response was obtained to the compounds chromatographed on the SE-30 column at lower isothermal temperatures than on the Dexsil column. At the attenuation shown in Fig. 2b (A and B), the minimal quantitatable amounts by the procedure approximated 250 pg for PE and 2 ng for PETN. However at higher attenuation the practical limit for quantitation was in the picogram range for all the compounds.

REFERENCES

1 R. W. BARNES, *J. Chromatogr.*, 31 (1967) 606.
2 D. B. PARIHAR, S. P. SHARMA AND K. K. VERMA, *J. Chromatogr.*, 31 (1967) 551.
3 V. HANKONYI AND V. KARAS-GASPAREC, *Anal. Chem.*, 41 (1969) 1849.
4 F. K. BELL, *J. Pharm. Sci.*, 53 (1964) 752.
5 E. SARNOFF, *J. Ass. Offic. Agr. Chem.*, 39 (1956) 630.
6 J. HANNSON, *Explosivstoffe*, 11 (1963) 73.
7 S. K. YASUDA, *J. Chromatogr.*, 51 (1970) 253.
8 J. A. KOHLBECK, *Anal. Chem.*, 37 (1965) 1283.
9 F. J. DICARLO, J. M. HARTIGAN AND G. E. PHILIPS, *Anal. Chem.*, 36 (1964) 2301.
10 I. W. F. DAVIDSON, H. S. MILLER AND F. J. DICARLO, *J. Pharm. Exp. Therap.*, 175 (1970) 42.
11 E. CAMERA AND D. PRAVISANI, *Anal. Chem.*, 36 (1964) 2109.
12 A. F. WILLIAMS AND W. J. MURRAY, *Nature*, 21 (1966) 816.