*Journal of Chromatography*, 110 (1975) 375–380


CHROM. 8328

# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PENTAERYTHRITOL IN PLASMA

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(Received March 24th, 1975)

## SUMMARY

A sensitive analysis of pentaerythritol in plasma has been devised, based on the formation of its tetra-*p*-methoxybenzoate derivative and high-performance liquid chromatography employing an ultraviolet photometric detector. The method permits analysis of pentaerythritol in the ppm range.

## INTRODUCTION

A program aimed at study of the bioavailability and metabolism of organic nitrates in man has commenced in these laboratories. One compound of interest, pentaerythritol tetranitrate (PETN), has recently been classified as being only "possibly" effective in the treatment and prevention of anginal attacks[1,2]. In humans, PETN is extensively converted to pentaerythritol dinitrate, pentaerythritol mononitrate, and pentaerythritol[3,4]. The latter material appears as the principal metabolite in blood. It was desired, as a component of our broader investigations, to study the absorption, distribution and elimination of pentaerythritol in humans. This project required a sensitive procedure for the determination of pentaerythritol in plasma.

Plasma analysis of $^{14}C$-labelled pentaerythritol by scintillation counting has been reported by Davidson *et al.*[3,4]. This method suffers from the disadvantages associated with administration of a radiolabelled compound to humans. PETN and its lesser nitrates have been sensitively measured by gas chromatography with electron capture detection[5]. A procedure utilizing trifluoroacetylation allegedly permits application of the method to pentaerythritol as well. However, in our hands, complete derivatization of pentaerythritol could not readily be effected. When gas chromatographed as such, pentaerythritol was found to satisfactorily develop on SE-30 and OV-17 columns at temperatures below 200°; however, insufficient sensitivity with flame ionization detection was anticipated.

Recently, Porcaro and Shubiak[6] reported a method for analyzing hexachlorophene based on reaction with *p*-methoxybenzoyl cloride and high-performance liquid chromatography (HPLC) of the resulting ester derivative. Since compounds containing the *p*-methoxybenzoate chromophore absorb strongly at 254 nm ($\varepsilon$ generally around 10,000 per *p*-methoxybenzoate unit), detection in the nanogram range is possible when measurement is effected with a simple low-pressure mercury photometric detector (possessing a suitable filter to permit operation at the 253.6-nm emission line of mercury). It was proposed that HPLC analysis of pentaerythritol as its tetra-*p*-methoxybenzoate might provide a sensitive procedure for determination of the former compound in plasma.

## EXPERIMENTAL

Pentaerythritol, obtained commercially (Aldrich, Milwaukee, Wisc., U.S.A.), was recrystallized from acetone–water prior to use. High-purity (distilled in glass) chloroform (Burdick and Jackson Labs., Muskegon, Mich., U.S.A.), was employed in all HPLC procedures. All other solvents and reagents were reagent grade.

### *Chromatographic system and operating conditions*

The liquid chromatograph (Waters ALC 202) was equipped with a low-pressure mercury (254-nm) photometric detector and fitted with a 0.6-m × 2.0-mm-I.D. stainless-steel column packed with a spherical siliceous porous layer pellicular material, 37–50 $\mu$m in diameter (Waters, Corasil II). The column was eluted at 1 ml/min with *n*-heptane–chloroform (3:2) at room temperature. Injections were performed with a 10-$\mu$l syringe (Hamilton 701N).

### *Preparation of reference pentaerythritol tetra-p-methoxybenzoate*

Pentaerythritol (3.42 g; 0.025 mole) in 50 ml of pyridine was reacted with *p*-methoxybenzoyl chloride (17.0 g; 0.1 mole) at room temperature for 16 h. The reaction mixture was poured into 200 ml of ice-water and extracted with three 200-ml portions of ethyl acetate. The combined ethyl acetate extract was washed successively with 5% sodium bicarbonate, 10% hydrochloric acid, water, and saturated sodium chloride solution, and then dried over anhydrous sodium sulfate. The ethyl acetate extract was reduced to dryness *in vacuo* to give a 90% yield of a thick yellowish oil. Preparative HPLC on a 1.2-m × 8-mm-I.D. stainless-steel column packed with a porous silica gel (Waters Porasil) gave the desired product as a white crystalline solid, m.p. 49–51°; UV ($CHCl_3$): $\lambda_{max}$ = 258 nm ($\varepsilon$ = 52,500); IR ($CHCl_3$): $\nu_{max}$ = 1715 $cm^{-1}$ (C=O) and 1260 $cm^{-1}$ (C–O); NMR ($CDCl_3$): $\delta$ = 3.84 (s, 12H, $OCH_3$), 4.64 (s, 8H, O–$CH_2$–), 6.87 (d, 8H, J = 9 Hz, aryl protons *ortho* to $OCH_3$), 7.94 (d, 8H, J = 9 Hz, aryl protons *ortho* to C=O); single spot at $R_F$ = 0.79 on a 250-$\mu$m silica gel $GF_{254}$ plate (Analtech, Newark, Del., U.S.A.) developed with benzene–methanol (9:1).

*Analysis.* Calculated for $C_{37}H_{36}O_{12}$: C, 66.06; H, 5.39. Found: C, 66.14; H, 5.59.

### *Procedure*

A 5-ml plasma sample (obtained from heparinized blood) is mixed with 2 ml

of 10% trichloroacetic acid and centrifuged at 2000 *g* for 15 min. The supernatant is decanted into a 35-ml glass-stoppered tube and treated with 2 ml of 10% sodium hydroxide, 0.7 ml of *p*-methoxybenzoyl chloride and 4 ml of *n*-heptane–chloroform (3:2). The resulting mixture is shaken for 60 min in a water-bath at 40°. Duplicate 5-$\mu$l portions of the organic layer are chromatographed and peak areas ($H \times W_{\frac{1}{2}}$) are determined for the pentaerythritol tetra-*p*-methoxybenzoate. The concentration of pentaerythritol in the sample is determined from the standard curve.

*Standard calibration curve*

Pooled plasma samples (5 ml) containing the following concentrations of pentaerythritol are prepared in duplicate: 2, 10 and 20 $\mu$g/ml. These are treated as indicated under *Procedure* and the areas obtained from the *p*-methoxybenzoate derivative are plotted against the pentaerythritol concentration. An $r$ value equal or greater than 0.990 should be produced.

## RESULTS AND DISCUSSION

Experiments were performed to determine the potential usefulness of liquid–liquid extraction in recovering pentaerythritol from biological media. At room temperature, pentaerythritol was found to be essentially insoluble in most common water-immiscible solvents. Solutions of 0.001 % could be produced in chloroform, yet this, the best solvent of those investigated, provided less than 10% recoveries of pentaerythritol from aqueous media even when multiple extraction and salting-out techniques were used. Additional recovery experiments were performed with acetone and $C_1$–$C_3$ alcohols/saturated carbonate biphasic combinations[7]. However, these too provided poor recoveries of pentaerythritol from aqueous solutions. Davidson *et al.*[3] extracted pentaerythritol with ethyl acetate–methanol (1:1) from urine specimens following reduction of the latter to dryness under a stream of nitrogen. This alternative recovery–extraction technique was rejected because it was deemed to be too time consuming. Further difficulty was experienced when a pyridine-catalyzed derivatization technique was utilized as it might be with residues that would result from a liquid–liquid or liquid–solid extraction step. That is, repeated chromatographic development of reaction mixtures containing excess *p*-methoxybenzoyl chloride in pyridine changed the performance characteristics (reductions in retention times of *p*-methoxybenzoate derivatives) of the Corasil II columns initially utilized. It was suspected that acylation of OH sites on these columns was being effected thereby causing the observed effect. Alternatively, pyridine and/or *p*-methoxybenzoyl chloride may adversely affect the "activation" of siliceous columns via some physical adsorption mechanism. Thus, while quantitative conversion of pentaerythritol to its tetra-*p*-methoxybenzoate can be readily effected with pyridine-*p*-methoxybenzoyl chloride mixtures, there was a serious drawback associated with the routine use of this derivatization method.

Porcaro and Shubiak[6] employed a Schotten–Baumann technique in preparing the *p*-methoxybenzoate derivative of hexachlorophene. It was proposed that a similar procedure might serve the purpose of not only affecting derivatization of pentaerythritol but because it could be performed in aqueous media, it would aid in the ultimate recovery of pentaerythritol (as its derivative) by liquid–liquid extraction. A combina-

tion derivatization–extraction method patterned after the literature procedure[6] is described in Experimental. The method employs a biphasic reaction mixture including an extracting solvent combination of identical composition to that found to satisfactorily chromatograph the pentaerythritol derivative. Using this method, the conversion–of pentaerythritol to its tetra-*p*-methoxybenzoate was found to proceed as indicated in Fig. 1. The curves in Fig. 1 point to an optimum temperature for derivatization–recovery of pentaerythritol. At 23°, the reaction proceeds too slowly while at 55°, yields are poor, probably due to excess hydrolysis of the *p*-methoxybenzoyl chloride reagent. At 40°, the reaction is essentially finished after 1 h. In water, yields (after 1 h) of pentaerythritol tetra-*p*-methoxybenzoate are typically 96–100% while in plasma yields are generally 55–60% (R.S.D. = 9–10%; $N$ = 7–9).

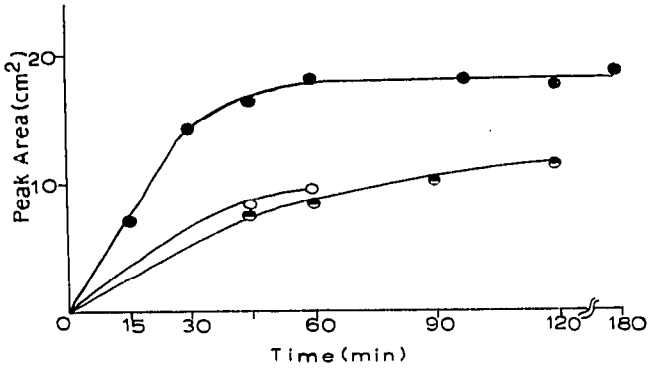


Fig. 1. Formation of pentaerythritol tetra-*p*-methoxybenzoate as a function of temperature. ◓—◓, 23°; ○—○, 55°; ●—●, 40°.

Several solvent system–internal phase combinations were evaluated for the liquid chromatographic development of pentaerythritol tetra-*p*-methoxybenzoate. Satisfactory development was observed with Corasil II developed with combinations of chloroform and *n*-heptane and on reversed-phase columns (Waters Corasil/$C_{18}$ and Corasil/phenyl, 37–50 μm) developed with mixtures of acetonitrile or methanol and water. For routine use, Corasil II used with *n*-heptane–chloroform (3:2) was the most conveniently utilized and gave superior resolution of the pentaerythritol derivative from native (derivatized) materials co-extracted from plasma. An illustrative chromatogram is depicted in Fig. 2.

A number of polyhydric alcohols were evaluated for possible use as internal standards in the determination of pentaerythritol in plasma. Of these, arabitol, 1,2-cyclohexanediol, digitoxose, erythritol, glycerol, inositol, 1,5-pentanediol, and xylitol appeared to form single derivatives in fair to good yields. All of these derivatives, however, possessed retention times that were excessively long or developed coincidently with the derivative of pentaerythritol or a native eluting peak. Consequently, a procedure employing an external standard was pursued. As indicated in Fig. 3, standard curves possessing correlation coefficients of 0.990 or greater could be produced from analyses performed with spiked pooled plasma samples. When this type of standard was utilized for analyses of pentaerythritol in individual plasma samples, data as indicated in Table I were achieved. The satisfactory accuracy and repro-

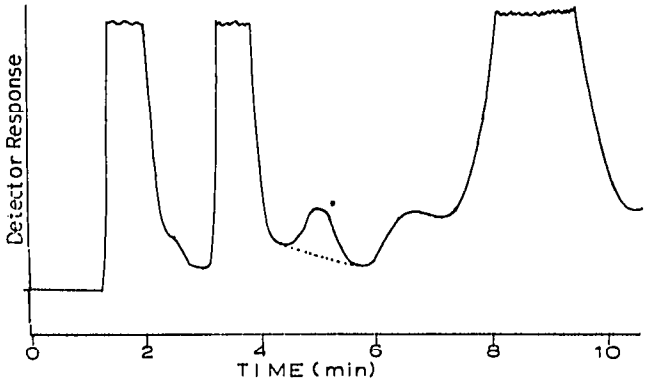


Fig. 2. Chromatogram obtained from analysis of a 5-ml plasma sample containing 6 μg/ml of pentaerythritol. An amount of pentaerythritol tetra-*p*-methoxybenzoate (*) equivalent to 37.5 ng of pentaerythritol was developed. Detector sensitivity, 08. The dotted line indicates the position of the baseline during the analysis of the blank plasma sample.

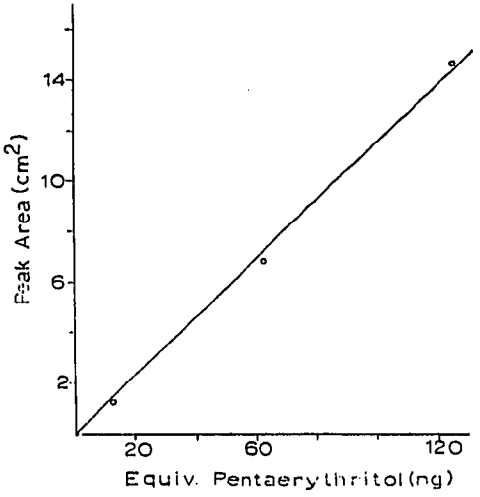


Fig. 3. Typical standard curve obtained from spiked pooled plasma samples. Each point represents an average of two to three determinations. Correlation coefficient, 0.990.

TABLE I

ACCURACY AND PRECISION OF HPLC ASSAY OF PENTAERYTHRITOL IN PLASMA

| *Amount (μg) added to 5-ml plasma samples* | *Recovery (%)* |
|---|---|
| 6.4 | 88.5 |
| 12 | 96.5 |
| 16 | 81.3 |
| 50 | 96.4 |
| 50 | 93.1 |
| 50 | 91.9 |
| 50 | 107.6 |
| 100 | 86.8 |
| Mean | 92.8 |
| R.S.D. | 8.5 |

ducibility of the method provides ready determination of ppm levels of pentaerythritol in plasma.

## ACKNOWLEDGEMENT

This work was supported by a grant from Marion Laboratories, Kansas City, Mo., U.S.A.

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