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Gas-Liquid Chromatographic Determination of Pantothenates and Panthenol

A. R. PROSSER and A. J. SHEPPARD

Abstract □ A procedure has been developed for the identification and quantitative determination of the pantothenates and panthenol by GLC. The pantothenate salts were converted to an ethyl ester by treatment with anhydrous ethanolic hydrogen chloride. The acetate derivatives of the pantothenic acid ethyl ester and panthenol were prepared by acetylation of the hydroxyl groups with a pyridine:acetic anhydride mixture. The excess reagents were removed and the oily residue was taken up in chloroform and injected on a 2.438-m. (8-ft.) × 4-mm. column packed with 2 % neopentyl glycol sebacate (NPGSb). Trimethylsilyl and trifluoroacetate derivatives of the pantothenates and panthenol were also analyzed by gas chromatography. The procedure is very sensitive; as little as 0.25 mcg. can be detected. Structures of the acetate derivatives were confirmed by IR and NMR spectroscopy.

Keyphrases □ Panthenates, panthenol—separation, determination □ Derivatives—pantothenates, panthenol □ GLC—separation, analysis □ IR spectrophotometry—structure □ NMR spectroscopy—structure

A number of satisfactory methods are available for the identification and determination of the pantothenates and pantothenyl alcohol (panthenol) (1–7). However, previous reports (8–15) have shown that some of the water-soluble vitamins are amenable to GLC, thus suggesting that panthenol, pantothenic acid, and the pantothenic acid salts could also be determined by this technique.

Unmodified polyhydroxy and carboxylic organic compounds, such as panthenol and pantothenic acid and its salts, are not volatile enough for direct GLC, and both have a tendency to decompose at high temperatures or are not soluble enough in the common organic solvents. However, these problems are easily overcome by converting the compounds to volatile derivatives such as acetates (AC), trimethylsilyl ethers (TMSE), or trifluoroacetates (TFA) for GLC analysis.

This paper describes the GLC determination of the acetate derivatives of pantothenic acid ethyl ester and panthenol. The acetyl derivatives (acetates) were chosen because of their relatively high degree of volatility and stability and their ease of preparation and cleanup. The ethyl ester of pantothenic acid was chosen rather than the methyl ester so that both derivatives would have equal carbon numbers and molecular weights; thus the separation would be based on the arrangement of the constituents within the molecule. Other derivatives such as trimethylsilyl ether, trifluoroacetate, and propionate were investigated and are discussed.

EXPERIMENTAL

Instrumentation—A gas chromatograph[1] fitted with a high-temperature hydrogen-flame ionization detector (FID) and a β-argon ionization detector (AID) with a 56-μc. ^{226}Ra foil was used with a 5-mv., 2-sec., 27.94-cm. (11-in.) strip chart recorder.

Materials—The following were used: *d*-pantothenyl alcohol, *d*-pantothenic acid calcium salt, and *dl*-pantothenyl alcohol (Sigma Chemical Co., St. Louis, Mo.); *d*-pantothenic acid calcium salt, trifluoroacetic anhydride (TFAA), propionic anhydride, and valeric anhydride (Eastman Organic Chemicals, Distillation Products Industries, Rochester, N. Y.); *dl*-pantothenic acid (K & K Laboratories, Inc., Plain View, N. Y.); *d*-pantothenic acid sodium salt (Mann Research Laboratories, Div. of Becton Dickinson & Co., New York, N. Y.); bis(trimethylsilyl)acetamide (BSA) (Perco Supplies, San Gabriel, Calif.); bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco, Inc., Bellefonte, Pa.); trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) (Applied Science Laboratories, Inc., State College, Pa.); and chloroform, (Baker analyzed reagents/spectrophotometric, J. T. Baker Chemical Co. Phillipsburg, N. J.). All other solvents and reagents used were reagent grade and required no further purification.

Preparation of Derivatives—*Esterification*—The methyl and ethyl esters of pantothenic acid were prepared from calcium pantothenate and sodium pantothenate; the procedure of Stoffel *et al.* (16) was used for the preparation of the methanolic and ethanolic HCl solutions. In a typical experiment, 54.4 mg. of calcium pantothenate (or its equivalent of sodium pantothenate) and 5 ml. of 2.5% (w/w) ethanolic HCl were stirred in a small round-bottom flask with a stirring bar for 1.5 hr. at room temperature. The ethanolic HCl solution was removed by flash evaporation.

Acetylation—The pantothenic acid ethyl ester (ethyl pantothenate) and panthenol were converted to the acetate derivatives by treating each compound with a 1:1 mixture of acetic anhydride and pyridine.

Panthenol (50.0 mg.) and/or pantothenic acid ethyl ester (obtained from the above described esterification of 54.4 mg. of calcium pantothenate) was placed in a small round-bottom flask containing a stirring bar and 3 ml. each of acetic anhydride and pyridine were added. The reaction mixture was stirred for 1 hr. at room temperature. The acetylating reagents were removed by flash evaporation at 40–50°. If the reaction product was to be used for spectroscopic

[1] Model 5000 series, Barber-Colman, Rockford, Ill.

studies, the residue was taken up in 2.5 ml. chloroform (spectrograde) and filtered; if the reaction product was to be used for gas chromatography only, the residue was diluted to 10 ml. with chloroform and filtered, and a suitable aliquot was injected directly into the chromatograph.

Standard Solutions—Primary GLC standards were prepared as outlined above except that the quantities of starting materials and reagents were increased and the reaction products were diluted to 25 ml. To prepare the esters, 500 mg. of panthenol and 543.5 mg. of calcium pantothenate were used with 25 ml. of ethanolic HCl; to prepare the acetate derivatives, 20 ml. of the acetic anhydride-pyridine reagent was used. The esterification step can be omitted in the preparation of panthenol triacetate when panthenol is the only compound to be analyzed. However, both starting materials were included in the same reaction vessel for the two reaction steps so that the primary GLC standard would contain both derivatives. Such a standard has advantages when peak identification as well as quantitative determination is required. Aliquots of the primary standard were diluted with chloroform to provide working standards so that a 2-μl. standardized injection would contain 0.5, 1.0, 1.5, 2.5, 5.0, 7.5, 10.0, or 15.0 mcg. of each derivative expressed as pantothenic acid and panthenol.

The derivatives for the spectroscopic studies were prepared individually in the same manner as outlined above for primary GLC standards and were also diluted to 25 ml. These primary standards for spectroscopy contained 20 mg./ml. of pantothenic acid (from 21.74 mg. of calcium pantothenate) as ethyl pantothenate diacetate and 20 mg./ml. of panthenol as panthenol triacetate.

GLC—Four different column packings were evaluated for their usefulness to resolve the derivatives of pantothenic acid and panthenol in this study. Column 1: 244 × 0.3 cm. i.d. packed with a biphase mixture of 2.5% neopentyl glycol succinate (NPGS) and 10% SE-30 (w/w) on 100/120 mesh diatomaceous earth.[2] Operating conditions for the separation of pantothenic acid ethyl ester diacetate and panthenol triacetate were: column 225°; detector, 280°; injector, 280°; and carrier gas, 50 ml./min. Column 2: 244 × 0.4 cm. packed with a liquid phase of 2% NPGSb (w/w) on 110/120 mesh Anakrom ABS for the analysis of pyridoxol triacetate, pantothenic acid ethyl ester diacetate, and panthenol triacetate. Operating conditions were: column, 230°; detector, 280°; injector, 280°; and carrier gas, 60 ml./min. Column 3: 183 × 0.4 cm. i.d. packed with 3% XE-60 (w/w) on 110/120 mesh Anakrom ABS for the analysis of pyridoxol (TMSE), pantothenic acid ethyl ester (TMSE), and panthenol (TMSE). Operating conditions were: column, 170°; detector, 250°; injector, 250°; and carrier gas, 60 ml./min. Column 4: 183 × 0.4 cm. i.d. packed with a liquid phase of 3% OV-17 (w/w) on 100/120 diatomaceous earth for the analysis of the TFA derivatives of panthenol and methyl pantothenate. Operating conditions were: column, 135°; detector, 260°; injector, 260°; and carrier gas 60 ml./min.

As in work reported previously (14), all columns were U-shaped Pyrex glass[3] and were preconditioned 24 hr. at 235° with carrier-gas flow rate of 60 ml./min. Argon was used as the carrier gas throughout the study. Compressed air and hydrogen flow rates were 460 and 36 ml./min., respectively, with the FID. A d.c. cell voltage of 900 was used at all times with the AID. Electrometer output was 1×10^{-10} amp. when FID was used. The electrometer output is given as full scale output into a 5-mv. recorder unless otherwise stated in the text. Peak areas were determined by triangulation (width at half-height × height = peak area).

RESULTS AND DISCUSSION

Structure of Derivatives—The structure of the derivatives was determined by IR and NMR spectroscopy. The IR data were obtained by dissolving the individual derivatives in spectro grade chloroform (20 mg./ml.) in a 0.05-mm. fixed KBr cell or preparing them as KBr wafers; a grating IR spectrophotometer[4] was used. No bands were observed in the expected region for hydroxyl groups. The following bands appeared in both derivatives and were almost identical: a band at 3,440 cm.$^{-1}$, which is indicative of secondary amides; the acetate ester band at 1,740 cm.$^{-1}$ (C=O stretch); the amide I band at 1,680 cm.$^{-1}$ (C=O stretch vib.); and the amide II band at 1,520 cm.$^{-1}$ (combination band of NH def. vib. and C—N str. vib.). The CH_3 band at 1,380 cm.$^{-1}$ for ethyl pantothenate diacetate was more intense than the 1,380 cm.$^{-1}$ band for panthenol triacetate, which indicates the presence of at least one more nonacetate CH_3 group.

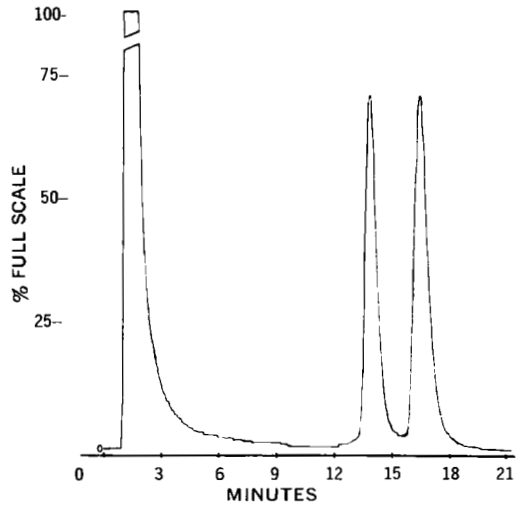

Figure 1—*Chromatogram of 2.16 mcg. of calcium pantothenate as ethyl pantothenate diacetate (14 min.) and 2.0 mcg. of panthenol as the triacetate (17 min.) in chloroform on a 2.5% NPGS-10% SE-30 biphase column at 225°.*

The presence of this CH_3 group can only be attributed to the CH_3 portion of the ethyl group in ethyl pantothenate diacetate. The band at 1,460 cm.$^{-1}$ had almost disappeared. The asymmetric and symmetric C—O—C stretch at 1,230 and 1,040 cm.$^{-1}$, respectively, are not significantly different for distinguishing the ethyl pantothenate diacetate from the panthenol triacetate (tri-*O*-acetyl panthenol).

The NMR spectral data were obtained from solutions of the ethyl pantothenate diacetate and panthenol triacetate in $CDCl_3$; a NMR spectrometer[5] was used. Tetramethylsilane was used as the reference standard. Chemical shifts are given in terms of δ (p.p.m.). The proton signals from the three —O-acetyl (acetate) groups of panthenol triacetate appeared at δ 2.04 (six protons) and δ 2.14 (three protons) as singlets. The proton from the —NH-group appeared at δ 7.26 as a triplet. The protons from the two —O-acetyl (acetate) groups of ethyl pantothenate diacetate appeared at δ 2.04 (three protons) and δ 2.13 (three protons) as singlets. The protons from the CH_3 portion of the ethyl group appeared at δ 1.25 (three protons) as a triplet. The protons from the CH_2 portion of the ethyl group appeared at δ 4.14 (two protons) as a quartet. These data indicate that the derivatives conform to the structures as stated in the nomenclature.

Efficiency of Derivative Preparation—The recovery of the pantothenate derivatives, expressed as mean ± standard deviation of six gravimetric determinations, was 92 ± 1.7%. GLC scans of the derivatives indicated no contaminants. The yield of panthenol triacetate was 96.3 ± 1.4%, with no indication of impurities by GLC analysis. The reaction efficiency may possibly be improved by converting the pantothenates to pantothenic acid and treating the acid with diazomethane.

Effect of Immobile Phase—The elution peaks of the pantothenates and panthenol derivatives gave distinct peaks on a biphase of the 2.5% NPGS + 10% SE-30 column at 225° (Fig. 1). The 2% NPGSb column at 230° and 60 ml./min. gave very good elution peaks with smaller quantities than the biphase column for the pantothenate and panthenol derivatives (Fig. 2). The 2.0% NPGSb column at 230° and 55 ml./min. gave excellent elution peaks for pyridoxol triacetate, pantothenic acid ethyl ester diacetate, and panthenol triacetate (Fig. 3). The fact that this column can be used to detect and quantitatively determine pyridoxol, pantothenates, and panthenol derivatives in the same analysis, and that niacin derivatives have been chromatographed on the same column (14) indicates that perhaps several more of the water-soluble vitamins can be

[2] Gas Chrom Q, Applied Science Laboratories, State College, Pa.
[3] Pyrex Glass Brand No. 7740, Corning Glass Works, Corning, N. Y.
[4] Perkin-Elmer model 337.
[5] Varian Model A-60.

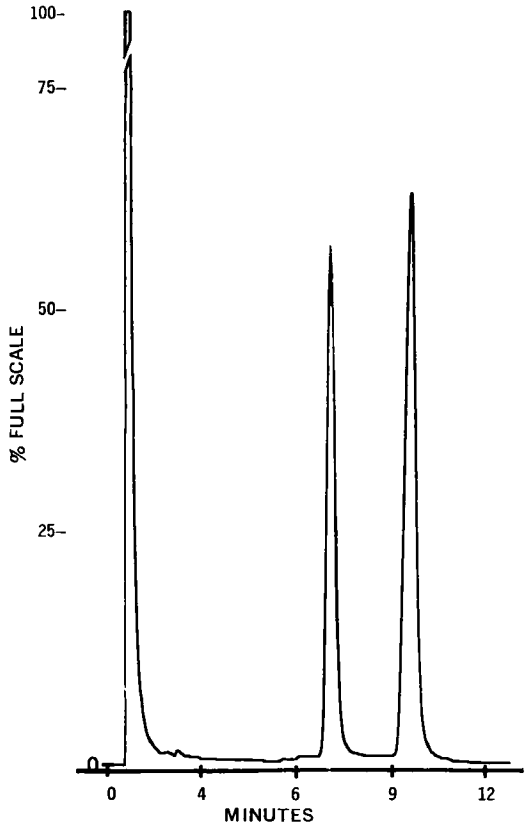

Figure 2—*Chromatogram of 1.0 mcg./μl. of calcium pantothenate as ethyl pantothenate diacetate (7.0 min.) and 1.0 mcg./μl. of panthenol as the triacetate (10.0 min.) in chloroform on a 2.0% NPGSb column at 230°.*

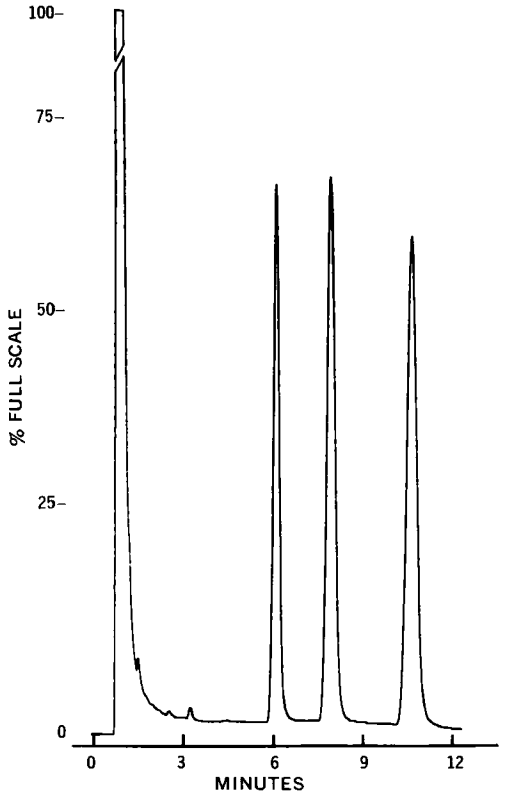

Figure 3—*Chromatogram of 0.60 mcg./μl. of pyridoxol as the triacetate (6.0 min.), 1.08 mcg./μl. of calcium pantothenate as ethyl pantothenate diacetate (8.0 min.), and 1.0 mcg./μl. of panthenol as the triacetate (11.0 min.) in chloroform on a 2.0% NPGSb column at 230°.*

chromatographed on the same column within a reasonable retention time.

Effect of Quantity Injected—Response data for the derivatives of panthenol triacetate and the pantothenate (ethyl ester) diacetate with the 2% NPGSb column are given in Table I, and show that the response was linear as the quantity of compound was increased.

Sensitivity—The GLC method for the pantothenates and panthenol is very sensitive. By using the FID, an attenuator setting of 1, an electrometer output of 1×10^{-9} amp., and a 0–1-mv. recorder, 5–8 ng. of panthenol and the pantothenates were detectable. The AID, with an attenuation of 1 and an electrometer output of 3×10^{-7} amp. detected 10 and 15 ng. of the panthenol and pantothenate derivatives, respectively. All quantities are based on weight of starting materials. Quantitation at these low levels is difficult because of the noise-to-signal ratio and there is less control over the quantity injected.

Other Derivatives—TMSE derivatives of pyridoxol, panthenol, and pantothenic acid ethyl ester were prepared by adding either acetonitrile–BSA–TMCS (5:5:1) or acetonitrile–BSTFA–TMCS (5:5:1) to these three compounds and heating at 50° for 30 min. (17). The derivatives were injected directly on the 3% XE-60 columns. The retention times were: pyridoxol (TMS), 4.3 min.; panthenol (TMS), 6.0 min.; and pantothenic acid ethyl ester (TMS), 12.5 min. There were three distinct and symmetrical peaks with no contamination peaks apparent.

The TFA derivatives were prepared (17) by treating equimolar mixtures of panthenol and pantothenic acid methyl ester with 2 ml. of TFAA for 45 min. at room temperature. The derivatives were diluted with acetonitrile and injected directly on the 3% OV-17 column. The retention times were: panthenol (TFA), 4.0 min., and pantothenic acid methyl ester (TFA), 6.0 min.

Propionate and valerate derivatives of panthenol and ethyl pantothenate were chromatographed on the 2% NPGS column. The peaks appeared in the order of their molecular weights. Although the peaks were symmetrical, they had considerably longer retention times than the acetate derivatives under the same operating conditions. The response was lower for the higher molecular weight derivatives than for the acetate derivatives.

Table I—Response of Pantothenic Acid Ethyl Ester Diacetate and Panthenol Triacetate[a]

mcg.	Peak Area,[b] mm.[2] Pantothenic Acid Ethyl Ester Diacetate	Panthenol Triacetate
0.5	11.0 ± 0.2	14.0 ± 0.2
1.0	25.0 ± 0.8	30.3 ± 0.4
1.5	38.4 ± 1.3	44.7 ± 0.5
2.5	60.3 ± 1.5	72.3 ± 0.9
5.0	129.8 ± 2.4	150.4 ± 1.7
7.5	200.0 ± 2.7	243.6 ± 2.5
10.0	275.2 ± 3.1	325.3 ± 2.8
15.0	412.3 ± 3.7	480.5 ± 3.2

[a] 10X attenuation, electrometer output 1×10^{-10} amp., hydrogen flame detector, and a 2.0% NPGSb column. [b] Mean of six analyses ± standard deviation.

CONCLUSIONS

GLC can be used to detect and analyze pantothenic acid salts and panthenol as acetyl derivatives, and offers a unique advantage in speed of separation, sensitivity, and convenience. Preliminary results indicate that trimethylsilyl ether and trifluoroacetate derivatives are also amenable to GLC analysis and that GLC analysis of the acetate derivatives are adaptable to pharmaceutical products. Studies are now underway on applying the GLC analysis to pharmaceuticals containing pantothenates and panthenol. This method may also find use as an analytical tool for studies such as those to determine the metabolic disposition of pantothenic acid or its analogs.

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Effect of Complex Formation on Drug Absorption X: Effect of Polysorbate 80 on the Permeability of Biologic Membranes

JUDITH A. ANELLO and GERHARD LEVY*

Abstract □ Low concentrations of a nonionic surfactant (polysorbate 80) in the bathing solution increase significantly the absorption and exsorption rate constants of 4-aminoantipyrine in goldfish. This effect is evident in absorption and exsorption studies involving chemical assays as well as in experiments in which absorption rate was determined indirectly on the basis of the time of onset of a pharmacologic effect. The ratio of rate constants with : without surfactant was similar in the three types of experiments. It is concluded that polysorbate 80 enhances drug transfer by a direct effect on the biologic membranes and not by interacting with the drug.

Keyphrases □ Complex formation—drug absorption □ Polysorbate 80 effect—membrane permeability □ Absorption, exsorption rates—goldfish □ Overturn time, goldfish—biologic assay □ Colorimetric analysis—spectrophotometry

Studies in this laboratory have shown that polysorbate 80, in concentrations below the critical micelle concentration, significantly increases the absorption of secobarbital and pentobarbital in goldfish (1). This effect was thought to be due either to the formation of a nonmicellar drug-surfactant complex which is absorbed more rapidly than the drug itself, or to a modification of the permeability characteristics of the biologic membranes by the surfactant (1). Subsequent studies, in which the absorption kinetics were determined on fish which had been immersed in surfactant solution, rinsed, and then placed in surfactant-free drug solution, revealed that enhanced absorption could also be obtained by pretreatment of the fish with polysorbate 80 (2). These observations suggested strongly that the surfactant exerts a direct permeability enhancing effect on the biologic membranes. However, the possibility could not be excluded that the enhanced absorption involves an interaction of the drug with surfactant molecules that are adsorbed on the membrane surface. It was believed that studies of drug transfer out of the fish (exsorption), where the surfactant is in the bathing solution, would provide a more definitive indication of the mechanism of the surfactant effect since drug and surfactant are then on opposite sides of the membrane. Also, since previous studies were based on pharmacologic effect data only, it was considered desirable to determine absorption rates by direct chemical assay as well as on the basis of the onset of a defined pharmacological effect. The experimental approach for the absorption and exsorption studies was as previously described by Levy and Miller (3), using 4-aminoantipyrine, a drug that is not measurably metabolized or protein bound in goldfish under the experimental conditions (4).

EXPERIMENTAL

Goldfish, *Carassius auratus*, common variety, weighing 10 g. on the average, were used. All fish in a given experiment were from the same lot.

Materials—Polysorbate 80 (lot No. 586 Atlas Powder Company), 4-aminoantipyrine (Eastman Organic Chemicals); Tris(hydroxymethyl) aminomethane (Tris) (Nutritional Biochemicals Corp.); and glycine (Eastman Organic Chemicals).

The drugs were dissolved in 0.05 *M* Tris or 0.05 *M* glycine buffer and the solutions were adjusted to pH 7.0 and 4.0, respectively, with hydrochloric acid.

Determination of Absorption and Exsorption Rates—The method has been described previously (3) except for the following modifications. In the absorption experiments, five fish were placed simultaneously in 1 l. of drug solution for designated times, then rinsed quickly in distilled water and stored in individual containers in a freezer until assayed. The fish were never kept frozen for more