*Journal of Chromatography*, 502 (1990) 401–406
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 173

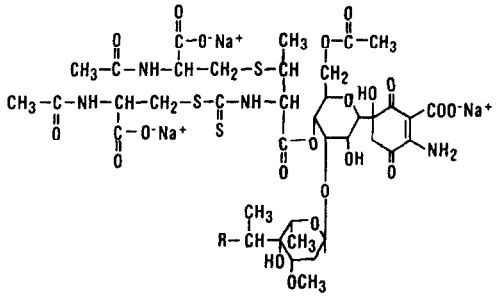
# Note

## Estimation of molecular isomer ratios of paldimycin sodium (U-70,138F) by gas chromatography

JOHN D. STODOLA* and PATRICK A. HARTMAN
*The Upjohn Company, Product Control Analytical Development, 4821-259-12, Kalamazoo, MI 49001 (U.S.A.)*

(First received August 2nd, 1989; revised manuscript received November 21st, 1989)

Paldimycin sodium (U-70,138F) is a mixture of trisodium salts of the N-acetyl-L-cysteine derivatives of fermentation derived paulomycin isomers (U-43,120)[1–3]. A previous report[4] described a gradient reversed-phase high-performance liquid chromatography (HPLC) method for the separation of paldimycin A (U-67,963) from paldimycin B (U-67,964) and from the corresponding paulomycins. The HPLC method is not capable of resolving paldimycin A from paldimycin $A_2$ (U-77,904) nor the corresponding B isomer from $B_2$ isomer (U-77,940) (see structures in Fig. 1). A method for determining the relative ratios of these isomers was desired to support



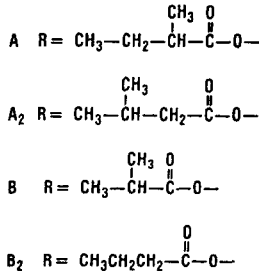


Fig. 1. Structures of isomers of paldimycin sodium. A = 2-methylbutyric acid, $A_2$ = 3-methylbutyric acid, B = 2-methylpropionic acid, $B_2$ = butyric acid.

the characterization of the bulk drug. The isomers arise[1,2] from variation in the butyric and methylbutyric acids linked via an ester bond at the C-7′ position of the sugar ring (Fig. 1). Previous reports of the separation of these and other short chain carboxylic acids include gas chromatography (GC)[5–14], HPLC[15–18], ion chromatography[17,19] and electrophoresis[20–21]. These reported methods were developed for the separation of the carboxylic acids as components which are not covalently bonded to the samples of interest however. Our goal was to develop a method which would cleave the relevant ester bonds and liberate the characteristic carboxylic acids in a medium which would allow subsequent separation by gas chromatography and indirect quantitation of the molecular isomer ratios of paldimycin sodium bulk drug.

## EXPERIMENTAL

### *Materials*

Paldimycin sodium samples were produced by Upjohn. Carboxylic acid standards and 2-vinylfuran were Gold Label quality (Aldrich, Milwaukee, WI, U.S.A.). HPLC-grade methanol and acetone (Burdick & Jackson, Muskegon, MI, U.S.A.) and Milli-Q water (Millipore, Milford, MA, U.S.A.) were used to prepare solutions. Concentrated phosphoric acid and concentrated ammonium hydroxide (Mallinckrodt, St. Louis, MO, U.S.A.) were used for adjustment of pH. High-purity compressed helium and hydrogen were delivered from gas cylinders (Welder, Kalamazoo, MI, U.S.A.) via copper tubing. Compressed air was supplied via copper tubing from an in-house system.

### *Instruments*

A Model 5890 gas chromatograph equipped with Model 7673A robotic sampler and flame ionization detector was used (Hewlett-Packard, Palo Alto, CA, U.S.A.). Glass columns (1–2 mm × 3 mm) manually packed with Carbowax 20M and 1% phoshoric acid coated on Graphpack GC 60–80 mesh (Alltech, Rockford, IL, U.S.A.) were used for separations. A 2-cm section at the head of each column was packed with 80–100 mesh Chromosorb 101 (Alltech) to act as a guard column. Other stationary phases surveyed include Porapak Q, Porapak QS (Waters Assoc., Milford, MA, U.S.A.), Chromsorb 101 and AT 1200 with phosphoric acid (Alltech) packed in (0.6–2 m × 3 mm) glass columns. GC–mass spectrometric (MS) studies were conducted on a VG-7070F MS system (Danvers, MA, U.S.A.) using electron ionization at 70 eV and scanning a mass-to-charge ratio of 440 to 20 at 1 s/decade of mass. The mass spectrometer was interfaced via a jet separator to a Model 5790 GC system (Hewlett-Packard, Palo, Alto, CA, U.S.A.) using a 1.5-m column operated isothermally at 150°C and 30 ml/min flow.

### *Preparations, conditions and calculations*

Samples and standards were diluted in a solution of concentrated phosphoric acid–acetone–Milli-Q water (1:10:90, v/v/v), Carboxylic acid standards were accurately prepared by serial dilutions at 0.1 mg/ml for 2-methyl propionic acid and 2-methylbutyric acid and at about 0.05 mg/ml for *n*-butyric acid and 3-methylbutyric acid. Paldimycin sodium samples (*ca.* 10 mg) were dissolved in *ca.* 200 μl of concentrated ammonium hydroxide–water (1:99, v/v) and diluted with 2 ml of the

acidic sample diluent. The sample diluent was injected as a blank solution in each run. The instrumental parameters were: column temperature 120°C, injector temperature 190°C, detector temperature 220°C, flame ionization detection (FID) hydrogen flow 50 ml/min, FID air/oxygen flow 200 ml/min, helium carrier gas flow 40 ml/min, injection volume 1 μl. The mole percent of each isomer was calculated via area percent using adjusted areas for the B isomer acids where the response factor was calculated as the ratio of the molecular weights.

*Validation*

Multiple injections of sample diluent blank, individual acid standards, freshly prepared samples and solution samples stored at room temperature for 16 h were injected to verify specificity, selectivity, precision and ruggedness. Accurately prepared solutions of reference standards and samples were injected to evaluate recovery, linearity and potential bias.

## RESULTS AND DISCUSSION

*Preparations*

Initial preparation of paldimycin sodium samples in a small volume of dilute ammonium hydroxide was necessary to ensure complete dissolution before dilution with the acidic sample diluent. Acetone was included in the sample diluent to maintain solubility of the carboxylic acid reference standards. The sample preparations must be acidic (apparent pH of 2 or less), because higher pH preparations yielded poor peak shapes and poor resolution of the acids of interest. Chemical degradation of the paldimycins in solution (as determined by HPLC) did not adversely affect the recovery of the acids of interest or generate any interfering peaks in the chromatograms. The method is not applicable to paulomycins[3], because they are insoluble in the sample diluent. Attempts to use methanol as an alternative solvent for paulomycins led to very poor chromatography and apparent stripping of the stationary phase.

*Specificity*

The Graphpak GC Carbowax 20M phase with phosphoric acid was specifically designed for separation of short-chain carboxylic acids[22]. It was the only phase studied (see Experimental) which provided resolution of 2-methylbutyric acid and 3-methylbutyric acid from isomers A and $A_2$. Similar separations of these two acids on phosphoric acid-treated stationary phases have been reported elsewhere[9,14]. Separation of 2-methylpropionic acid and butyric acid from isomers B and $B_2$ was also obtained as demonstrated by the chromatograms of the sample solvent blank, reference standards and a paldimycin sodium bulk drug sample in Fig. 2. The acids of interest are resolved witin 18 min. No interfering peaks were detected in the blank chromatogram. An additional peak was detected in chromatograms of all preparations of paldimycin sodium (Figs. 2 and 3) however. The separation of this peak from the carboxylic acids of interest was dependent on column length, with the best resolution obtained with a 1.5-m column. GC–MS studies suggest this peak elutes with a retention time and mass spectrum matching those of 2-vinylfuran (*m/e* values of 94, 65, 39). The structure and mechanism by which this species is formed has not been rigorously established, but it is speculated that it may involve high-temperature

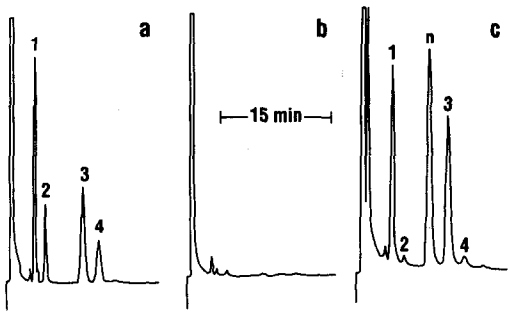



Fig. 2. Chromatograms of (a) reference standard preparation, (b) blank and (c) paldimycin sodium bulk drug sample preparation. See Experimental for conditions. Peaks: 1 = 2-methylpropionic acid; 2 = butyric acid; 3 = 2-methylbutyric acid; 4 = 3-methylbutyric acid; n = peak from paldimycin sodium preparations (matches 2-vinylfuran).

decomposition and/or dehydration of a sugar portion of the paldimycin structure. Fig. 3 shows injections of preparations of isolated paldimycin isomers to further demonstrate the specificity of the chromatography.

Paldimycins C and D derived from the corresponding paulomycins[23] would be expected to lead to observation of *n*-propionic acid and acetic acid respectively with this method. Propionic acid was not detected in any of the samples tested, however, with a detection limit of *ca.* 0.1% indicating the absence of paldimycin C. Acetic acid which may have been contributed by paldimycin D would be masked by the acetic acid liberated from the acetate esters on the other rings and N-acetyl-L-Cys (Fig. 1) and would elute near or in the solvent front and is therefore not quantitated by this method. Paldimycins E and F[23] do not contain ester side chains and are also consequently not detectable by this method.

*Quantitation*

The expected equivalents of each carboxylic acid were recovered from accurately weighed paldimycin sodium samples by comparison to accurately prepared carboxylic

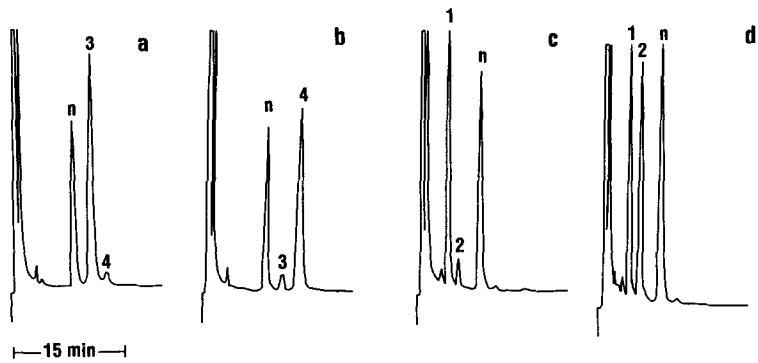



Fig. 3. Chromatograms of paldimycin sodium isomers (a) paldimycin A, (b) paldimycin $A_2$, (c) paldimycin B and (d) paldimycin B and $B_2$. See Experimental for conditions, Fig. 1 for structures and Fig. 2 for peak identification.

TABLE I

COMPARISON OF GC ISOMER AND HPLC ISOMER RESULTS FOR PALDIMYCIN SODIUM LOTS

GC results listed parenthetically are for the minor isomers. N.D. = Not detected. See Fig. 3 for chromatograms of the individual isomers listed at the bottom of this table.

| *Sample* | *HPLC* | | *GC* | |
|---|---|---|---|---|
| | *A+A₂* | *B+B₂* | *A+A₂ (A₂)* | *B+B₂ (B₂)* |
| Bulk drug U | 62.3 | 37.7 | 64.6 (3.1) | 35.4 (0.6) |
| Bulk drug V | 57.5 | 42.5 | 59.5 (2.3) | 40.5 (0.3) |
| Bulk drug W | 59.7 | 40.3 | 61.7 (2.4) | 38.3 (0.4) |
| Bulk drug X | 63.8 | 36.2 | 65.9 (2.8) | 34.1 (0.7) |
| Bulk drug Y | 63.6 | 36.4 | 65.6 (2.7) | 34.4 (0.6) |
| Bulk drug Z | 56.2 | 43.8 | 58.9 (3.5) | 41.1 (0.3) |
| Paldimycin A | 100 | N.D. | 99.9 (4.0) | 0.1 (N.D.) |
| Paldimycin B | 1 | 99 | 0.8 (N.D.) | 99.1 (5.8) |
| Paldimycin $A_2$ | 100 | N.D. | 100 (6.3) | < 0.1 |
| Paldimycin $B+B_2$ | 1 | 99 | 0.9 (N.D.) | 99.1 (45.9) |

acid reference standard preparations. The combination of the acidic sample preparation and high temperature in the injector port is therefore adequate to ensure quantitative cleavage of the esters at C-7′. Linearity of area response was verified by injection of bulk drug prepared over a range of 25%–125% of the suggested assay concentration. Plots of amount detected *versus* amount added gave correlation coefficients of greater than 0.999. Intercepts were not significantly different from zero indicating the absence of bias.

The major isomers A and B were quantitated with a relative standard deviation (R.S.D.) of about 1% and the minor isomers $A_2$ and $B_2$ with an R.S.D. of about 10% for eight independent preparations. Table I lists results for several bulk drug lots and purified paldimycin isomers and compares the summations of $A+A_2$ and $B+B_2$ isomers determined by HPLC[4] to those determined by this method. The GC results for the minor isomer content are listed parenthetically in the table. The $A_2$ and $B_2$ isomers constitute less than 4% and 1% respectively of the isomers detected in the bulk drug lots studied.

## CONCLUSIONS

A GC method for determining paldimycin sodium isomer ratios has been validated. It utilizes an acidic sample preparation and *in situ* ester cleavage within a GC system which gives linear response, good precision and is selective for carboxylic acids arising from paldimycins A, $A_2$, B and $B_2$.

## ACKNOWLEDGEMENTS

We thank A. D. Argoudelis, J. M. Timko and W. J. Haak for bulk drug samples, J. DeZwaan for helpful discussions and W. K. Duholke for the GC–MS study.

REFERENCES

1 A. D. Argoudelis, L. Baczynskyj, J. A. Buege, V. P. Marshall, S. A. Mizsak and P. F. Wiley, *J. Antibiot.*, 40 (1987) 408–418.
2 A. D. Argoudelis, L. Baczynskyj, S. A. Mizsak, F. B. Shilliday, P. A. Spinelli and J. DeZwaan, *J. Antibiot.*, 40 (1987) 419–436.
3 A. D. Argoudelis, T. A. Brinkley, T. F. Brodasky, J. A. Buege, H. F. Meyer and S. A. Miszak, *J. Antibiot.*, 35 (1985) 285–294.
4 P. A. Hartman and J. D. Stodola, *J. Chromatogr.*, 385 (1985) 363–368.
5 P. G. Brotz and D. M. Schaefer, *J. Microbiol. Methods*, 6 (1987) 139–144.
6 N. P. Bole and R. K. Colwell, *J. Chromatogr.*, 410 (1987) 190–194.
7 J. L. Berdauge, *Lait*, 66 (1986) 233–246.
8 W. R. Thomann and G. B. Hill, *J. Clin. Microbiol.*, 23 (1986) 392–394.
9 M. Brazier, G. DeSmet, F. Pieri, P. Daniel, *Clin. Chim. Acta*, 148 (1985) 261–265.
10 D. N. Buchanan and J. G. Thorne, *Clin. Chim. Acta*, 145 (1985) 183–191.
11 R. J. Wiggins, M. Wilks and S. Tabaguchi, *J. Clin. Pathol.*, 38 (1985) 933–936.
12 M. J. Playne, *J. Sci. Food Agric.*, 36 (1985) 638–644.
13 I. Molnár-Perl, V. Fabian-Vonsik and M. Pinter-Szakacs, *Chromatographia*, 18 (1984) 673–676.
14 A. Tangerman, A. van Schaik, M. T. Meuwese-Arende and J. H. M. van Tongeren, *Clin. Chim. Acta*, 133 (1983) 341–348.
15 D. Tusseau and C. Benoit, *J. Chromatogr.*, 395 (1985) 323–333.
16 R. W. Gillett and G. P. Ayers, *Anal. Chim. Acta*, 177 (1985) 273–277.
17 R. Schwarzenbach, *J. Chromatogr.*, 251 (1982) 339–358.
18 R. L. Patience and J. D. Thomas, *J. Chromatogr.*, 249 (1982) 183–186.
19 K. Kihara, S. Rokushita and H. Hatano, *J. Chromatogr.*, 410 (1987) 103–110.
20 X. Huang, J. A. Luckey, M. J. Gordon and R. N. Zare, *Anal. Chem.*, 61 (1988) 766–770.
21 T. Barth, *Anal. Chem.*, 59 (1987) 2232–2237.
22 *Catalogue No. 50*, Alltech Associates, Rockford, 1983, p. 12.
23 A. D. Argoudelis, L. Baczynskyj, W. J. Haak, W. M. Knoll. S. A. Mizsak and F. B. Shilliday, *J. Antibiot.*, 41 (1988) 157–169.