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Note

Gradient reversed-phase high-performance liquid chromatographic separation of paldimycin (U-70,138) antibiotics and related compounds

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Paulomycin (I in Fig. 1) is a mixture of antibiotics produced by *Streptomyces paulus* and effective against several strains of gram positive bacteria[1]. Paldimycin (U-70,138) is the bis-N-acetyl-L-cysteine derivative of paulomycin[2] and is isolated as the trisodium salt (U-70,138F; II in Fig. 1). Paldimycin trisodium has more favorable aqueous solubility and pharmacokinetic properties than the native paulomycin[3]. The mono-N-acetyl-L-cysteine adducts are also potential impurities in the paldimycin. The structures of paulomycin and paldimycin trisodium are shown in Fig. 1. There are two molecular forms of each compound, designated A and B, differing by a

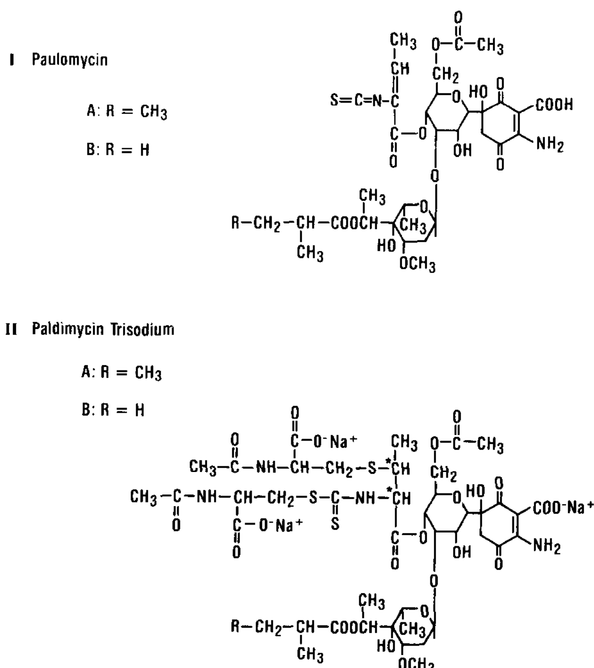

Fig. 1. Structures of paulomycin (I) and paldimycin trisodium (II). Stars indicate undefined asymmetric centers introduced by derivatization.

methylene group in the side chain, as illustrated. In addition, the bis-N-acetyl-L-cysteine derivatization produces two new asymmetric carbons in paldimycin, thus four stereoisomers for each molecular form are anticipated (Fig. 1).

In the development of a liquid chromatographic assay for paldimycin, it was desirable to separate not only the molecular forms but the stereoisomers as well. Other considerations and restrictions were also involved. Reversed-phase liquid chromatography with gradient elution was selected because of the vast range of polarity of the compounds studied. Potential instability of U-70,138 in solution required nearly neutral buffers and room temperature in order to minimize on-column degradation. The wide-pore reversed-phase columns were investigated because of their superior performance with larger molecules[4]. UV detection at 254 nm was suitable for these compounds.

EXPERIMENTAL

A modular liquid chromatographic system, consisting of a Series 4 gradient pump and controller and Model ISS-100 autosampler (Perkin-Elmer, Norwalk, CT, U.S.A.), 25 cm × 4.6 mm I.D. Zorbax 150-Å C_8 analytical column (DuPont, Wilmington, DE, U.S.A.), Spectroflow 773 UV detector (Kratos, Ramsey, NJ, U.S.A.), and Waters Model 840 chromatographic data system (Millipore, Milford, MA, U.S.A.) was used.

Mobile phase A consisted of: 0.02 *M* ammonium acetate (Mallinckrodt AR-grade, Paris, KY, U.S.A.), 0.01 *M* L-valine (Sigma, St. Louis, MO, U.S.A.), 950 ml/l Milli-Q Water (Millipore, Bedford, MA), and 50 ml/l acetonitrile (Burdick & Jackson, Muskegon, MI, U.S.A.) with an inadjusted pH of *ca.* 6.8. Mobile phase B was acetonitrile.

The gradient profile was

Initial	90% A	10% B
Linear 40 min to	80% A	20% B
Linear 40 min to	40% A	60% B
Return 2 min to	90% A	10% B
Reequilibrate 14 min at	90% A	10% B

at a constant flow-rate of 1.5 ml/min.

The sample concentration was *ca.* 5 mg/ml paldimycin in mobile phase A with 20-μl injections.

Triethylamine (TEA) (Eastman-Kodak, Rochester, NY, U.S.A.), ammonium monobasic phosphate (Mallinckrodt), and tetrahydrofuran (THF) (Burdick & Jackson) were also used in the optimization study. Other analytical HPLC columns tested included: μBondapak C_{18} (300 mm × 4.6 mm I.D., Waters, Milford, MA, U.S.A.), PRP-1 (150 mm × 4.6 mm I.D.; Hamilton, Reno, NV, U.S.A.), Bakerbond wide pore C_4 and C_8 (250 mm × 4.6 mm I.D.; J. T. Baker, Phillipsburg, NJ, U.S.A.), and the standard Zorbax C_8 (250 mm × 4.6 mm I.D.; DuPont).

RESULTS AND DISCUSSION

Specificity

The final separation of a synthetic mixture of each of the U-70,138 analogues using the optimized conditions is shown in Fig. 2. The order of elution was: a quartet of paldimycin B stereoisomers, a quartet of paldimycin A stereoisomers, single peaks for the mono-N-acetyl-L-cysteine B and A, respectively as only one isomer forms, followed by the parent paulomycins B and A, respectively. Near baseline resolution of the stereoisomers of paldimycin was obtained only when L-valine was in the mobile phase (Fig. 3). It is postulated that the optically active amino acid not only interacts as a competing amine or ion pairing agent but also creates a chiral environment on the packing; and this is the reason for the superior resolution of diastereomers not generally obtained with reversed-phase chromatography. Methods for reducing tailing via addition of amine mobile phase modifiers[5], chiral separations enhanced with amino acid modifiers[6] and reversed-phase diastereomer separations[7,8] have been reported. The L-valine reversed-phase system described appears to exhibit a combination of these effects for this application. Further studies are planned to better define the role of this and other amino acid modifiers in these separations.

Optimization

The effect of L-valine is further illustrated in the series of chromatograms shown in Fig. 3. Various combinations of ammonium acetate buffer (pH 7), TEA, and THF were used with and without the L-valine in mobile phase A. The same gradient profile of 10–20% B in 40 min was used in each case. As clearly seen in Fig. 3, optimum resolution and efficiency was obtained with only L-valine and the buffer. Dramatic changes in specificity were observed with the addition of either TEA or THF. Classical ion-pairing reagents, such as alkyl sulfonates or tetraalkylammonium salts, were not investigated. Equivalent separations were observed with either am-

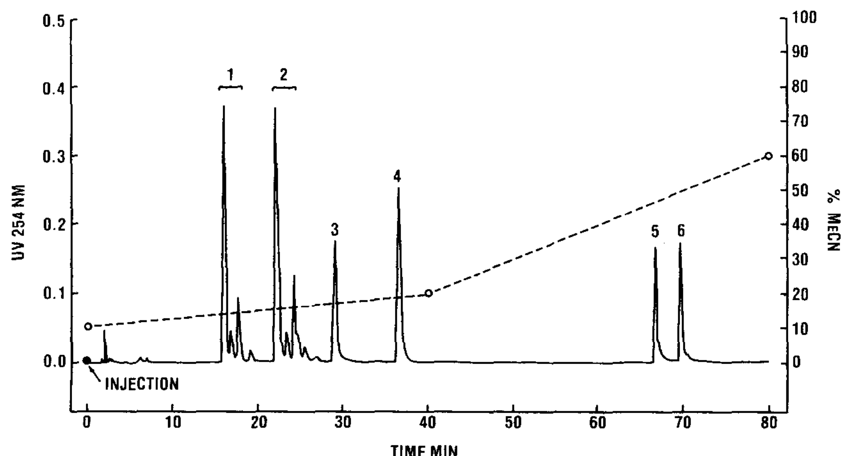

Fig. 2. Chromatogram of paldimycin isomers (U-70,138) and related compounds (liquid chromatographic conditions as in Experimental). MeCN is acetonitrile. 1 = Paldimycin B; 2 = paldimycin A; 3 = mono-adduct B; 4 = mono-adduct A; 5 = paulomycin B; 6 = paulomycin A.

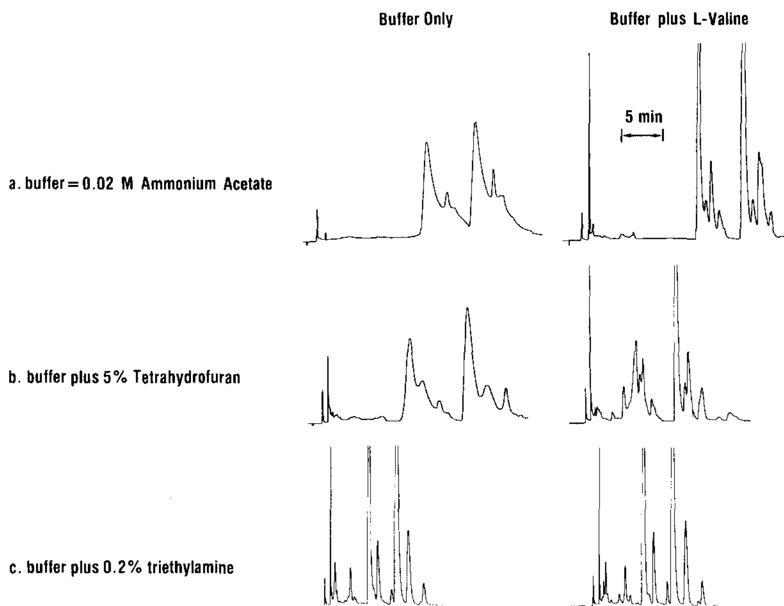

Fig. 3. Optimization of paldimycin isomer separation using L-valine as a mobile phase modifier (gradient slope 10–20% B in 40 min in each case).

monium acetate or phosphate buffers at pH 6.8, indicating no reliance on anion type. The buffer concentrations were optimized at 0.02 *M* ammonium acetate and 0.01 *M* L-valine. Less concentrated buffers did not provide the desired retention and resolution while more concentrated buffers also diminished the selectivity of the separation of the stereoisomers. The effect of the L-valine was noticeable on all of the columns listed in the Experimental section. The Zorbax 150-Å C_8 was the best choice since problems with severe tailing and inadequate resolution of the diastereomers were experienced with each of the other columns. The addition of TEA to mobile phase A did significantly reduce tailing, however, inferior resolution was still observed.

Various combinations of initial acetonitrile content and gradient slopes were tested. As is usually the case with larger molecules, the log k' (capacity factor) *versus* percent modifier plot had a steep slope for each of the paldimycin analogues. A change of about 5% acetonitrile made the difference between no retention and near total retention of U-70,138. Stadalius *et al.*[9] have demonstrated that the best gradient separations of larger molecules are achieved with relatively high flow-rates (>1 ml/min) and shallow gradient scopes ($>$ than 1% modifier/min). This was also the case for paldimycin. Minor changes in either the initial or final acetonitrile composition were sometimes required to compensate for variations due to instrument type, mobile phase preparation, column history, etc., but in all cases linear gradient slopes were used.

Linearity and precision

Linearity of detector response and reproducibility of the analytical method

TABLE I

LINEARITY OF RESPONSE FOR U-70,138 POTENCY ASSAY

Linear regression parameters: slope = 0.996; intercept = −0.002; corr. coeff. = 0.9999; estimated S.D. = 0.02; estimated S.D. slope = 0.002; estimated S.D. intercept = 0.01.

Percent of specified assay concentration	*Added (mg/ml)*	*Found (mg/ml)*	*Recovery (%)*
20	1.061	1.06	100.0
40	1.994	1.98	99.3
60	3.045	3.02	99.2
80	4.097	4.07	99.3
100*	4.988	4.988	100.0
100*	5.079	5.079	100.0
120	6.013	5.97	99.3
140	7.012	7.00	99.8
160	7.924	7.88	99.4
			$\bar{x}$ = 99.6% σ = 0.4 R.S.D. = 0.4%

* Standards.

TABLE II

ASSAY RESULTS FOR VARIOUS LOTS OF PALDIMYCIN TRISODIUM BULK DRUG

	Lot			
	A	*B*	*C*	*D*
Potency				
Form A	497 μg/mg	494 μg/mg	485 μg/mg	511 μg/mg
Form B	372 μg/mg	364 μg/mg	381 μg/mg	357 μg/mg
Total	869 μg/mg	858 μg/mg	866 μg/mg	868 μg/mg
Impurity				
Total mono-adducts	<0.1%	<0.1%	<0.1%	<0.1%
Total paulomycins	<0.1%	<0.1%	<0.1%	<0.1%
Isomer ratio				
A1	66.4%	64.9%	66.4%	66.5%
A2	6.9%	8.8%	7.1%	6.8%
A3	23.0%	22.6%	22.3%	22.7%
A4	3.7%	3.8%	4.1%	4.0%
B1	65.6%	65.4%	66.8%	66.8%
B2	9.4%	9.6%	9.6%	9.4%
B3	20.5%	20.5%	18.6%	19.6%
B4	4.5%	4.5%	5.0%	4.2%

were determined by assaying known concentrations of paldimycin bulk drug over the range of 20–160% of the specified assay concentration. These results are listed in Table I. The paldimycin potency values are expressed as the sum of the A and B diastereomers. No statistical bias was observed and the intra-assay precision was *ca.* 0.5% (relative standard deviation, R.S.D.).

Assay results

Data obtained by this method include the amount of each of the A and B forms of paldimycin (as the sum of the four diastereomers for each), their sum (for total potency), isomer ratios of the individual diastereomers (calculated by area percent), and an estimation of potential impurities (detected in the bulk drug sample). Representative results for typical lots are listed in Table II.

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