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High-performance liquid chromatographic separation of β-methyl ADC-13 enolphosphate diphenyl ester and its α-methyl diastereomer

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Abstract

The development of a high-performance liquid chromatographic (HPLC) separation of β-methyl ADC-13 enolphosphate diphenyl ester, an intermediate compound in the synthesis of a carbapenem antibiotic drug candidate, and its α-methyl diastereomer is reported. The method development involved separation on different columns in both normal- and reversed-phase modes. The use of normal-phase mode resulted in the desired elution order of the two diastereomers. The influence of different polar modifiers and their concentrations on resolution, capacity factor and separation factor was investigated. Different stationary phases were compared for their efficiency and resolution. The optimized separation was applied to the determination of the minor diastereomer in the bulk intermediate, and 0.1% minor diastereomer was detectable.

Keywords: Diastereomer separation; Stationary phases, LC; Mobile phase composition; Methyl ADC-13 enolphosphate diphenyl ester

1. Introduction

β-Methyl ADC-13 enolphosphate diphenyl ester (β-MEPDE) is an intermediate in the synthesis of a carbapenem antibiotic drug candidate. The β-MEPDE compound has four stereogenic centers as shown in Fig. 1. The carbon atom labelled with an asterisk in Fig. 1 is the last stereogenic center introduced, with an *R* configuration. During the introduction of this stereogenic center, partial racemization may occur resulting in the α-methyl diastereomer, α-methyl ADC-13 enolphosphate diphenyl ester (α-MEPDE), with an *S* configuration at this stereogenic center. Therefore, an analytical method was needed to separate the β-MEPDE from the α-MEPDE and determine the purity of the desired β-MEPDE diastereomer, in order to ensure the quality of the synthetic product.

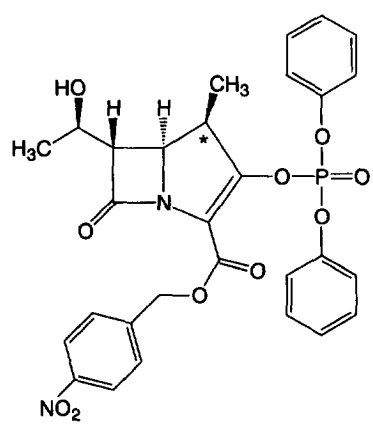

Fig. 1. Structure of β-methyl ADC-13 enolphosphate diphenyl ester (β-MEPDE).

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High-performance liquid chromatography (HPLC) is a powerful and widely used technique for the separation and quantitation of diastereomers. Several recently published papers [1–6] are just a few examples of the applications of HPLC in this area. Some of these works were done in reversed-phase mode on non-chiral columns, such as C_{18} or C_4 columns [1–3]. Others involved the use of chiral columns, such as cyclodextrin [4], 3,5-dimethylphenylcarbamates of cellulose and amylose [5], and Nucleosil Chiral-2 [3], in normal-phase mode. Gopal et al. [6] used quinine as a mobile phase additive to enhance the separation of diastereomers of a pharmaceutical compound on a diol column in normal-phase mode.

The present paper describes the development of a HPLC method for the separation of β-MEPDE and it α-methyl diastereomer on a non-chiral column. The method development involved the selection of a proper separation mode and optimization of mobile phase and stationary phase. The optimized normal-phase method was able to detect 0.1% minor diastereomer.

2. Experimental

2.1. Instrumentation

The chromatographic system consisted of a SpectraSystem P4000 HPLC pump, an AS3000 autosampler equipped with a 20 μl sample loop, and a UV2000 variable-wavelength UV detector (Thermal Separation Products, Piscataway, NJ, USA). Chromatograms were processed by a PE Nelson data system equipped with Access*Chrom software (version 1.9) (PE Nelson, Cupertino, CA, USA).

2.2. Material

All the mobile phase solvents were HPLC grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The sodium phosphate (monobasic) used for the preparation of aqueous buffer in the mobile phase was purchased from Sigma (St. Louis, MO, USA). The β-methyl ADC-13 enolphosphate diphenyl ester and its α-methyl diastereomer were provided by Process Research and Development Department of Merck Research Labs. (Rahway, NJ, USA).

2.3. Chromatographic conditions

The columns used in reversed-phase mode were a Zorbax Rx C_8 column (5 μm, 250×4.6 mm, Rockland Technologies, distributed by MAC-MOD Analytical, Chadds Ford, PA, USA), a YMC ODS-A column (5 μm, 250×4.6 mm, YMC, Wilmington, NC, USA) and a YMC ODS-AQ column (5 μm, 250×4.6 mm, YMC). The columns used in normal-phase mode were a Inertsil silica column (5 μm, 250×4.6 mm, MetaChem Technologies, Torrance, CA, USA), a Nucleosil diol column (7 μm, 250×4.6 mm, Alltech Associates, Deerfield, IL, USA), and a Zorbax CN column (5 μm, 250×4.6 mm, Rockland Technologies). In all cases, column temperature was maintained at 23°C. The mobile phases were isocratically pump-mixed at the specified compositions. In reversed-phase mode, the mobile phase was aqueous phosphate buffer (20 m*M*) at the specified pH, mixed with acetonitrile at the specified volume-to-volume ratio. In normal-phase mode, the mobile phase was hexane mixed with one of the polar modifiers, which were ethyl acetate and isopropanol (IPA), at the specified volume-to-volume ratio. The flow-rate was 1.0 ml/min in all cases. Detection was performed by UV at 270 nm. The capacity factor k' was determined as $k'=(t_R-t_0)/t_0$, where t_R and t_0 were retention times of retained and unretained compounds, respectively. In the normal-phase mode, the t_0 was determined by injecting pure hexane, which was a weaker solvent than the hexane–polar modifier mixture, and noting the time of appearance of the hexane peak [7].

2.4. Preparation of samples

For reversed-phase separations, the samples were dissolved in acetonitrile–water (50:50, v/v). For normal-phase separations, the samples were dissolved in the corresponding mobile phases.

3. Results and discussion

3.1. Separations in reversed-phase mode

Since diastereomers possess different physical properties, it is possible to separate them using a non-chiral column. The initial separation attempt was made in the reversed-phase mode. The use of a C_{18} column (YMC ODS-A) gave a partial separation at pH 6.0 (Fig. 2a). Adjustment of the pH to a lower value (3.1) did not improve the separation (Fig. 2b).

Another column selected was a YMC ODS-AQ column. This column had hydrophilic endcappings which could allow polar eluent to penetrate between the C_{18} chains to enhance solute-stationary phase interactions. Stronger retention and often different selectivity are encountered on ODS-AQ compared with conventionally endcapped ODS [8]. However, the result in the present study was not satisfactory (Fig. 2c). As expected, the retention times increased compared with those obtained on the conventional ODS column (Fig. 2a); but the separation actually became slightly worse.

A Zorbax C_8 column was also unable to provide satisfactory separation within a similar analysis time (Fig. 2d).

In all the reversed-phase separations, no baseline separation was achieved in less than 25 min, and the minor diastereomer (α-methyl diastereomer) eluted after the major one (β-methyl diastereomer). Because the injection of large quantity of sample was needed in order to detect the minor diastereomer at 0.1% level, the tail of the major diastereomer would cause interference to the detection of trace amount of minor diastereomer. Although it might be possible to further separate the two diastereomers by decreasing the organic content in the mobile phase, the expected longer analysis time would make the method undesirable. Therefore, the use of normal-phase mode to invert the elution order as well as to improve the separation would be a better choice.

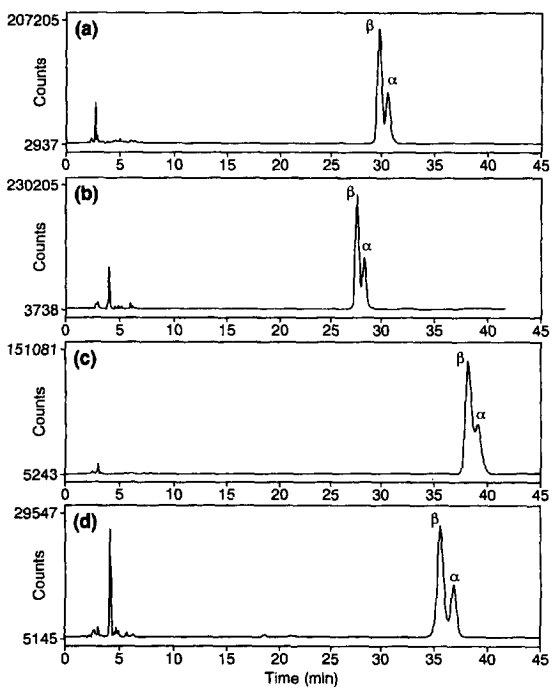

Fig. 2. Chromatograms of reversed-phase separations. (a) Column: YMC ODS-A; mobile phase: acetonitrile–aqueous buffer (pH 6.0) (50:50, v/v); (b) same as (a) except pH 3.1; (c) column: YMC ODS-AQ; other conditions: same as in (a); (d) column: Zorbax Rx C_8; mobile phase: (v/v) acetonitrile–aqueous buffer (pH 3.1) (45:55, v/v). Sample concentration: 0.16 mg/ml β-diastereomer and 0.068 mg/ml α-diastereomer for (a), (b) and (c), and 0.027 mg/ml β-diastereomer and 0.012 mg/ml α-diastereomer for (d).

3.2. Separations in normal-phase mode

3.2.1. Hexane-ethyl acetate as mobile phase

The commonly used columns for normal-phase separations include silica, diol and CN columns. With the use of a mixture of hexane and ethyl acetate as mobile phase, all three columns gave different degrees of separation (Fig. 3). Based on the knowledge gained from the reversed-phase separations, it was expected that the minor α-methyl diastereomer would elute before the β-methyl diastereomer in normal-phase mode. Indeed, the results proved this. Under the conditions noted in Fig. 3, baseline separation was achieved in less than 15 min on both the silica and the diol columns. The CN column did not give baseline separation within similar analysis time.

Table 1 compares the chromatographic parameters of the separations shown in Fig. 3. Among the three columns, the silica column yielded the highest separation factor and the best resolution in the shortest analysis time. The silica column also showed higher efficiency than the diol column. The larger particle size (7 μm) in the diol column was a

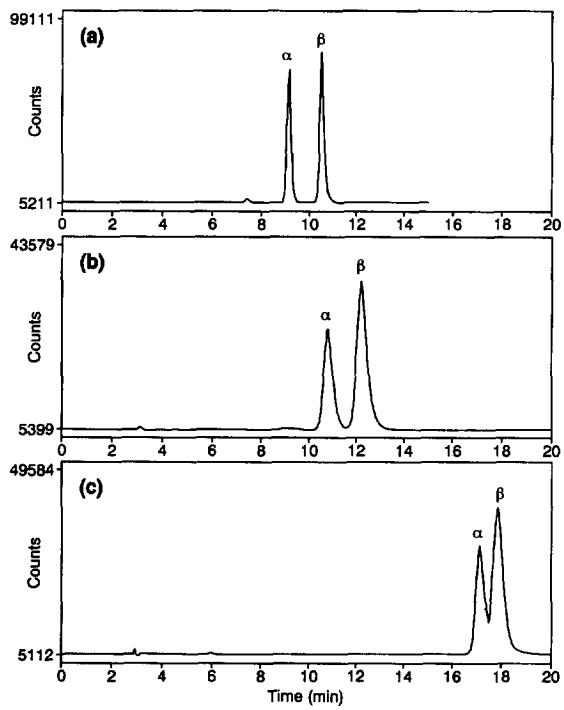

Fig. 3. Chromatograms of normal-phase separations using hexane–ethyl acetate as mobile phase. (a) Column: silica; mobile phase: hexane–ethyl acetate (40:60, v/v); (b) column: diol; mobile phase: hexane–ethyl acetate (50:50, v/v); (c) column: CN; mobile phase: hexane–ethyl acetate (75:25, v/v). Sample concentration: 0.030 mg/ml β-diastereomer and 0.022 mg/ml α-diastereomer.

contributing factor to the lower efficiency of the column. The broader peak obtained on the diol column would render a poorer detection limit. The CN column gave a resolution far from being satisfactory. Therefore, the silica column was the best choice.

The influence of ethyl acetate concentration in the mobile phase on capacity factor, resolution and separation factor was also studied on all three columns (see Table 2). The silica column gave baseline resolution and showed higher resolution and separation factor than the diol column at all the ethyl acetate concentrations studied. The CN column did not give a baseline resolution until the ethyl acetate concentration was lowered to 15% and the capacity factors were above 20.

The k' values in Table 2 gave a clear indication of the degrees of retention of the diastereomers on different columns in hexane–ethyl acetate system. At all the ethyl acetate concentrations common to both the silica and diol columns, each diastereomer showed stronger retention (larger k') on the silica column than on the diol column. The k' values on the CN column were less than 6.0 at 25% ethyl acetate concentration; however, the k' values on the diol column were above 14.0 at a higher ethyl acetate concentration (30%), indicating that the diol column gave stronger retention than the CN column. Therefore, the silica column gave the strongest retention and the CN column gave the weakest, when the mobile phase composition was maintained the same.

Compared with a diol column, a silica column is known to have stronger interaction with solutes through stronger hydrogen bondings [7,9]. Weiser et al. [10] reported that when a relatively weak solvent system was used, a CN column provided similar selectivity as a silica column, and the primary adsorption sites on the CN column were the residual silanol groups which provide hydrogen bondings; they observed weaker retentions on the CN column and attributed that to the shielding of the residual silanol groups by the adjacent cyano-propyl bonded phase. Therefore, the strongest retention on silica

Table 1
Comparison of chromatographic parameters of the diastereomers on different columns

Column	t_{R1} (min)	t_{R2} (min)	k'_1	k'_2	N_1	N_2	α	R_s
Silica	9.1	10.5	1.77	2.19	13330	13580	1.24	4.1
Diol	10.8	12.2	2.60	3.08	3450	3510	1.18	1.8
CN	17.1	17.9	5.12	5.38	–	–	1.05	0.99

Subscripts "1" and "2" represent α- and β-methyl diastereomers, respectively.
Chromatographic conditions are listed in Fig. 3. The number of theoretical plates was determined as $N = 16(t_R/W)^2$, where t_R is the retention time and W is the peak width. The W was measured by the computer software described in Section 2. The N values were not determined for CN column due to non-baseline separation.

Table 2
Influence of ethyl acetate concentration in the hexane–ethyl acetate mobile phase on capacity factors, resolution and separation factor on different columns

Ethyl acetate (%)	Silica column				Diol column				CN column			
	k'_1	k'_2	R_s	α	k'_1	k'_2	R_s	α	k'_1	k'_2	R_s	α
15									21.43	22.99	1.8	1.07
20									9.48	10.05	1.3	1.06
25									5.12	5.38	0.99	1.05
30					14.38	17.46	3.2	1.21				
35					8.08	9.69	2.4	1.20				
40	7.99	10.00	5.9	1.25	5.08	6.08	2.1	1.20				
45	5.20	6.51	5.5	1.25	3.37	4.00	1.9	1.19				
50	3.60	4.46	5.0	1.24	2.60	3.08	1.8	1.18				
55	2.48	3.07	4.6	1.24	1.84	2.17	1.7	1.18				
60	1.77	2.19	4.1	1.24	1.36	1.59	1.5	1.17				
65	1.28	1.59	3.6	1.24								

Subscripts "1" and "2" represent α- and β-methyl diastereomers, respectively.

column and the weakest retention on a CN column in the present study were expected; the results suggested that the hydrogen bondings were the strongest on the silica column and the weakest on the CN column. It also appeared that the stronger the hydrogen bondings provided by each column, the better the separation of the two diastereomers on that column; this suggested that the separation was mainly controlled by hydrogen bondings between the solutes and the stationary phases.

3.2.2. Hexane–isopropanol (IPA) as mobile phase

Another mobile phase system investigated was hexane–IPA. Fig. 4 shows the chromatograms of the diastereomers obtained on the three different columns. The silica column still performed the best, giving a baseline separation in less than 15 min. The diol column, however, did not yield a baseline separation within 30 min. The CN column showed a co-elution of the diastereomers in over 60 min.

The influence of IPA concentration in the mobile phase on capacity factor, resolution and separation factor was also studied on all three columns (see Table 3). By comparing the data in Tables 3 and 2, one can conclude that the hexane–IPA system gave poorer resolution and separation factor than the hexane–ethyl acetate system on all three columns, when the capacity factor was maintained at similar values.

One interesting fact was noted by comparing the capacity factors in the 10% IPA row in Table 3. In the hexane–IPA system, the CN column showed the largest k' and the silica column showed the smallest.

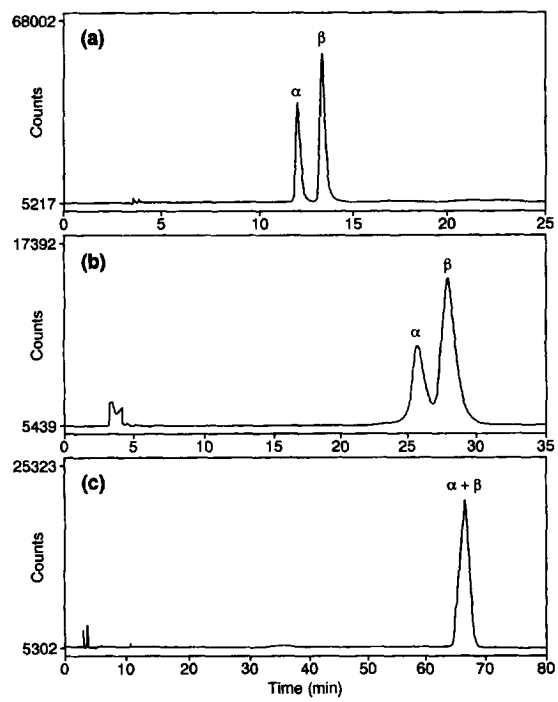

Fig. 4. Chromatograms of normal-phase separations using hexane–IPA (90:10, v/v) as mobile phase. Column: (a) silica; (b) diol; (c) CN. Sample concentration: 0.030 mg/ml β-diastereomer and 0.017 mg/ml α-diastereomer.

Table 3
Influence of IPA concentration in the hexane–IPA mobile phase on capacity factors, resolution and separation factor on different columns

IPA (%)	Silica column				Diol column				CN column			
	k'_1	k'_2	R_s	α	k'_1	k'_2	R_s	α	k'_1	k'_2	R_s	α
5	8.99	10.36	3.2	1.15	24.70	27.84	1.9	1.13				
10	2.64	3.04	2.5	1.15	7.61	8.34	1.2	1.10	22.76	22.76	0	1.00
15	1.35	1.55	2.0	1.15								
20	0.84	0.96	1.6	1.14	2.34	2.50	1.1	1.07				

Subscripts "1" and "2" represent α- and β-methyl diastereomers, respectively.

This order of k' on the columns was just the opposite of that in the hexane–ethyl acetate system.

One hypothesis to explain this phenomenon is that it could be due to the significant differences among the increases of adsorption energy of the polar mobile phase modifier on the different stationary phases. Snyder [11] showed that the k' of a solute increases with the increase of the net adsorption energy ΔE:

$$\Delta E = E_{xa} - mE_{sa} \quad (1)$$

where E_{xa} and E_{sa} refer to the adsorption energies of solute (x) and solvent (s) on the stationary phase, and m is a constant. When the polar modifier is changed from ethyl acetate to IPA, the E_{sa} is expected to increase due to stronger hydrogen bonding characteristics of IPA. A significantly larger increase of E_{sa} value on the silica column than that on the diol column could make the ΔE and k' on the silica column smaller than those on the diol column. The same logic can be used to explain the fact that the k' on the diol column became smaller than that on the CN column.

When a mobile phase system containing a more polar component such as an alcohol is used, the selectivity on CN column can be markedly different compared to that on silica column [10,12]. Weiser et al. [10] suggested that the more polar component in the mobile phase may lead to suppression of the effect of residual silanols in retention on CN column, leaving the cyano groups as the major adsorption sites, which interact with solutes through dipole–dipole interactions. In the present study, when hexane–IPA was used as mobile phase on the CN column, the loss of separation of the diastereomers could be hypothetically attributed to a lack of hydrogen bondings between the solutes and the residual silanol groups on the stationary phase. This also suggested that the separation of the diastereomers was mainly controlled by hydrogen bondings between the solutes and the stationary phases.

3.2.3. Application

Based on the results discussed above, it was concluded that the silica column with hexane–ethyl acetate (40:60, v/v) as mobile phase was the optimum system (Fig. 3a). The separation was achieved within a short analysis time (12 min). Although baseline resolution can be achieved within even shorter time on the silica column using a higher ethyl acetate content in the mobile phase, an analysis time shorter than 10 min was not preferred due to the concern about possible interference from other impurities.

The final application of this optimized separation was to determine trace amount of minor α-methyl diastereomer present in the bulk of β-methyl diastereomer. Therefore, it was necessary to determine the minimum detectable level of the minor diastereomer. This was determined by spiking different amounts of the minor diastereomer into the sample of pure β-methyl diastereomer. With the concentration of β-methyl diastereomer being 0.3 mg/ml, it was possible to detect 0.1% minor diastereomer (Fig. 5).

4. Conclusion

A normal-phase HPLC method was successfully developed for the separation of α- and β-methyl diastereomers of ADC-13 enolphosphate diphenyl ester on a non-chiral column. A silica column with

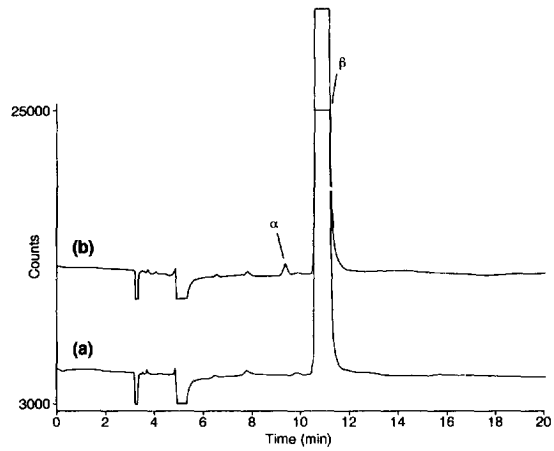

Fig. 5. Chromatograms of (a) pure β-methyl diastereomer and (b) pure β-methyl diastereomer spiked with 0.1% α-methyl diastereomer. Conditions as in Fig. 3a; sample concentration of β-methyl diastereomer: 0.3 mg/ml.

hexane–ethyl acetate as mobile phase gave the best performance. The use of normal-phase mode provided the desired elution order of the diastereomers. The separation appeared to be controlled by hydrogen bondings between the solutes and the stationary phases. The optimized method could detect 0.1% α-methyl diastereomer in the bulk of β-methyl diastereomer.

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