Investigation of Liquid and Gas Chromatography Techniques for Separation of Diastereomers of β-(α-Methylbenzyl) Amino Isobutyric Acid

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Abstract

Cryptophycins are macrolides investigated as potential anticancer agents. These large cyclic molecules are generated via a convergent process, utilizing the coupling of several smaller fragments synthesized individually. During early synthetic development of the β-amino acid fragment C, analytical methods are necessary for the characterization of products resulting from the various routes being studied. One route being evaluated produces (*RR*) and (*RS*) diastereomers of β-(α-methylbenzyl) amino isobutyric acid as intermediates. To measure diastereomeric excess (%de), assay conditions using high-performance liquid chromatography (HPLC) and capillary gas chromatographic (GC) techniques are explored. Derivatization methods using trifluoroacetyl- and silyl-derivatives are investigated for use with capillary GC. The results of the GC investigations are found to be only partially successful. Ion-pair HPLC is determined to be the optimal technique, utilizing pentanesulfonic acid as the counter ion to the amine group of β-(α-methylbenzyl) amino isobutyric acid.

Introduction

Cryptophycins are macrolides reported as potent tumor-selective cytotoxins originally isolated from some forms of blue-green algae (1). The total synthesis of various cryptophycin analogs has been accomplished through a convergent approach in which four smaller subunits, or fragments, are synthesized, coupled together, and then cyclized (2–5). The fragments have been referred to in the literature as A, B, C, and D (6). Cryptophycin A and the individual fragments making up the molecule are shown in Figure 1.

Fragment C in this case is (*R*)-3-amino-2-methylpropanoic acid (V). One route proposed for the synthesis of (V) is shown in Figure 2. This route was investigated with the potential of being diastereoselective (7). The reaction of methylacrylic acid (I) with (*R*)-α-methylbenzylamine (II) was expected to result in a mixture of diastereomers of β-(α-methylbenzyl) amino isobutyric acid (III, IV).

In order to optimize the synthesis, an assay to monitor the diastereomeric excess (%de) of β-(α-methylbenzyl) amino isobutyric acid was required. Authentic samples of β-(α-methylbenzyl) amino isobutyric acid were submitted for characterization and development. Both NMR and liquid chromatography (LC)–mass

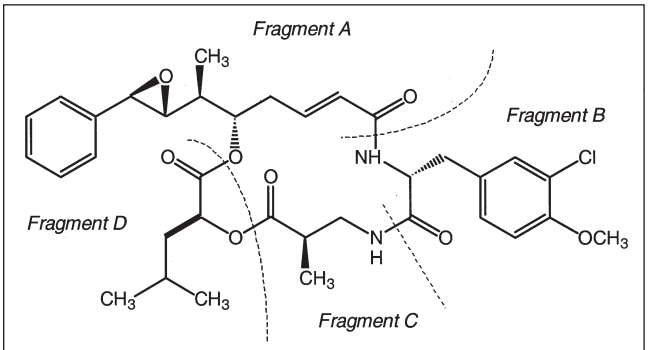

Figure 1. Cryprophycin A with fragments A, B, C, and D indicated.

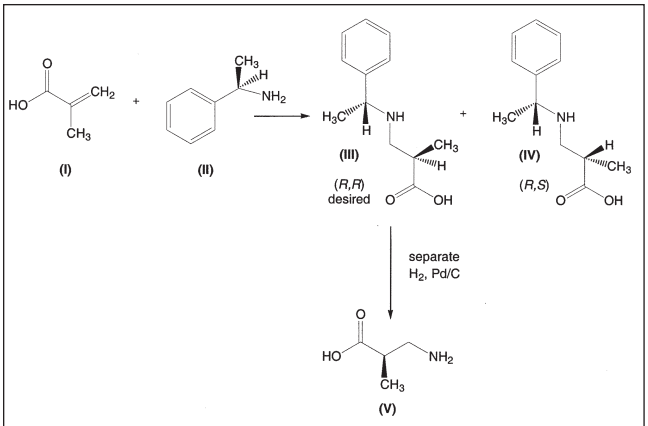

Figure 2. Synthesis of (*R*)-3-amino-2-methylpropanoic acid (V) via the formation of (*RR*) and (*RS*) methylbenzylamine diastereomers.

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spectrometry (MS) were used to verify the presence of β-(α-methylbenzyl) amino isobutyric acid in the samples (8). Although NMR studies indicated the presence of a mixture of *RR*- and *RS*-diastereomers in some samples, the initial LC–MS conditions did not resolve the diastereomers.

It was apparent that a more detailed examination of the separation was required. Because diastereomers differ in their physicochemical properties, they can be separated by conventional chromatography, such as reversed-phase (RP) high-performance LC (HPLC) and capillary gas chromatography (GC) (9,10). To help speed analytical development, both RP-HPLC and capillary GC techniques were investigated concurrently.

Experimental

Solvents and chemicals

HPLC-grade acetonitrile (ACN), methylene chloride, and tetrahydrofuran (THF) were obtained from Burdick & Jackson Laboratories (Muskegon, MI). Sodium phosphate dibasic and phosphoric acid (85%) were obtained from EM Science (Gibbstown, NJ). Quantities of *RR*- and *RS*-β-(α-methylbenzyl) amino isobutyric acid were synthesized for use during development, the authenticity being confirmed by proton NMR and LC–MS (Lilly Research Laboratories, Eli Lilly, Indianapolis, IN). *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) (Regisil RC-2) were obtained from Regis Technologies (Morton Grove, IL). Trifluoroacetic anhydride (99+%), triethylamine (99%), and 1-pentanesulfonic acid (PSA) sodium salt (98%) were obtained from Aldrich Chemical Company (Milwaukee, WI). Purified water produced by a Milli-Q filtration system (Millipore, Milford, MA) was used for all aqueous solutions. All other chemicals were of analytical-reagent grade.

Chromatographic equipment

HPLC equipment

HPLC chromatographic studies were carried out using a Shimadzu LC-10A HPLC system (Shimadzu, Columbia, MD) equipped with a SCL-10A system controller, LC-10AT pump, SPD-10A UV–vis detector, and SIL-10A autoinjector. The analytes were detected and quantitated by UV absorption at 254 nm. Injection volumes were 20 µL. Column flow rate and temperature were maintained at 1 mL/min and 50°C, respectively. Chromatographic separations were performed using either a Zorbax SB-Phenyl column (250- × 4.6-mm i.d., 5-µm particle size) or a Zorbax SB-C_8 column (250- × 4.6-mm i.d., 5-µm particle size) (MacMod Analytical, Chadds Ford, PA).

ACN and THF were utilized as strong solvents for isocratic and gradient elution. The aqueous mobile phase for both was 25mM sodium phosphate dibasic adjusted to pH 3.0 with phosphoric acid. The aqueous mobile phase used for investigation of ion-pair chromatography (IPC) consisted of either 2.5 or 5mM PSA and 25mM sodium phosphate dibasic adjusted to pH 2 with phosphoric acid. Data acquisition was provided by a Turbochrom chromatographic data system (PerkinElmer, Shelton, CT).

GC equipment

GC studies were carried out using both a Hewlett-Packard (Wilimington, DE) 5890 Series II capillary gas chromatograph equipped with flame ionization detector and a Hewlett-Packard 5890 Series II Plus capillary gas chromatograph coupled to a 5972 mass selective detector. Injections into the GCs were made in the split mode with a split ratio of 1:25 and an injector temperature of 250°C. A Hewlett-Packard 7673 autosampler was used for all injections. Helium was used as the carrier gas and was set to 1 mL/min (35 cm/s) at the initial oven temperature while operating in the constant pressure mode. The fused-silica capillary columns used for these investigations were the following: (*i*) DB-1 [100% dimethylpolysiloxane, 30-m × 0.25-mm i.d. column (df = 0.25 µm)], (*ii*) DB-Wax [polyethylene glycol, 30-m × 0.25-mm i.d. column (df = 0.25 µm)], and (*iii*) DB-210 [50% trifluoropropyl–methylpolysiloxane, 30-m × 0.25-mm i.d. column (df = 0.25 µm)]. All columns were obtained from J&W Scientific (Folsom, CA).

For experiments performed using the DB-1 stationary phase, the GC temperature program consisted of the following: an initial oven temperature of 40°C, held for 1 min, then ramped at 10°C/min up to 300°C, at which it was held for 30 min. The temperature program utilized with the DB-Wax column consisted of an initial temperature of 110°C held for 1 min, increased at 15°C/min up to 250°C, and then held for 30 min at 250°C. For those experiments that utilized the DB-210 stationary phase, the temperature program was the following: an initial oven temperature of 45°C held for 1 min, a 15°C/min ramp to 240°C, and then held at 240°C for 20 min.

The mass spectrometer was operated in the full-scan mode using a mass range of 33–550 amu at a rate of 1.48 scans/s. The heated capillary transfer line was set to 300°C. The instrument was tuned and calibrated prior to analysis with perfluorotributylamine (PFTBA). Data acquisition for GC experiments was performed using a Turbochrom chromatographic data system (PerkinElmer). For those experiments in which GC–MS was utilized, the data was acquired using a HP Chemstation (Hewlett-Packard).

Sample preparation

Trimethylsilyl (TMS) derivatives for GC analyses were prepared using BSTFA + 1% TMCS reagent. Approximately 1.5 mg of sample was weighed into a vial and diluted with 250 µL of BSTFA + 1% TMCS reagent. The sample was diluted to volume by the addition of 750 µL of ACN. The sample solution was then allowed to react with constant stirring for 20 min at 60°C. The resulting solution was injected neat onto the GC.

Acetyl derivatives for GC analyses were generated using trifluoroacetic anhydride (TFAA) reagent. Approximately 1.5 mg of sample was dissolved in 250 µL TFAA reagent. The solution was allowed to react while being sonicated for 20 min at room temperature. The sample was dried under a nitrogen stream and then reconstituted to 1 mL with methylene chloride for injection into the GC–MS.

All HPLC sample solutions were prepared by weight, at a concentration of approximately 1 mg/mL and diluted in the mobile phase being used.

Results and Discussion

GC assay development

Initial investigations to develop a method for determination of %de for β-(α-methylbenzyl) amino isobutyric acid involved a direct assay. The underivatized compound failed to elute using either a nonpolar dimethylpolysiloxane phase (DB-1) or a polar polyethylene glycol phase (DB-Wax). Failure of the diastereomers to elute was because of either low volatility resulting from strong intermolecular attractions between polar groups or adsorption effects in the column. GC analysis of molecules, which include polar functional groups (such as amines and acids), are often difficult to chromatograph because of their tendency to interact with active sites present in the stationary phases and supports. Replacement of active protons found on the polar functional groups with a neutral group, such as a TMS, can significantly reduce or eliminate detrimental hydrogen bonding, often resulting in improved chromatography (11). Derivatives of the acid and amine functional groups of amphoteric β-(α-methylbenzyl) amino isobutyric acid were studied as a means of improving general chromatographic behavior by reducing dipole interactions and hydrogen bonding.

In addition to improving chromatographic peak shape, derivatization was utilized to enhance intermolecular interactions between the stationary phase and analyte molecule. Common practice for separating chiral molecules on an achiral phase is by diasteroemeric derivatization. Several factors influence the effectiveness of these derivatizations in obtaining adequate separation, including changing the character of functional groups attached to the asymmetric center (12–14). In this case, the (*RR*) and (*RS*) diastereomers of β-(α-methylbenzyl) amino isobutyric acid were essentially derivatives of chiral (*R*)-3-amino-2-methylpropanoic acid. However, the differences were apparently not great enough to allow separation using the achiral phases being used. The β-(α-methylbenzyl) amino isobutyric acid was derivatized in order to maintain good chromatography and enhance the differences between the diastereomers and increase the prospects for separation.

TMS derivatives of β-(α-methylbenzyl) amino isobutyric acid were prepared using BSTFA + 1% TMCS. Although overall peak shape was improved significantly, the silyl-diastereomers were not resolved on the DB-1. A total-ion chromatogram (TIC) and accompanying electron impact (EI) spectra of a BSTFA derivative of β-(α-methylbenzyl) amino isobutyric acid is shown in Figure 3. The EI fragmentation pattern indicated that a monosilyl derivative was present, in which the TMS group was attached at the carboxyl group.

The conformation of β-(α-methylbenzyl) amino isobutyric acid was such that intramolecular hydrogen bonding between the carboxyl carbonyl and amine proton was possible because of rotation around the amine–carbon bonds. Substitution of the amine proton by a bulky group such as TMS was considered desirable for the elimination of intramolecular hydrogen bonding. It was thought that the addition of TMS to only the carboxyl group did not facilitate separation of the diastereomers because it could not block the hydrogen bonding or fix the conformation. The amine was apparently too hindered for the relatively large BSTFA and did not react under the mild conditions being used.

Acetyl derivatives were studied as well as the silyl analogs. TFAA

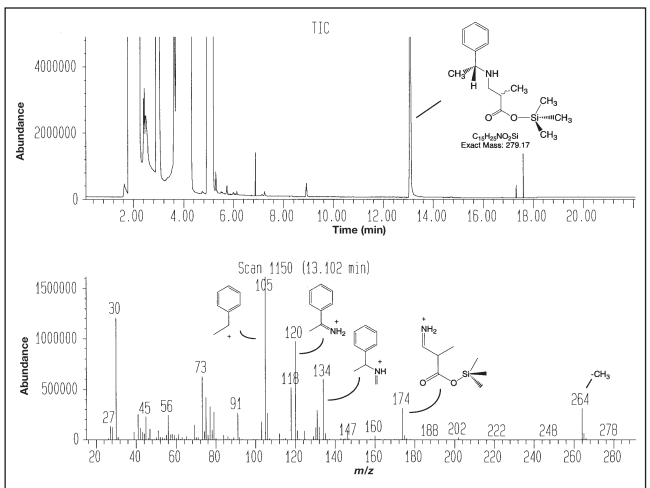

Figure 3. GC–MS TIC and EI spectra of silyl derivatives of diastereomers of β-(α-methylbenzyl) amino isobutyric acid (III).

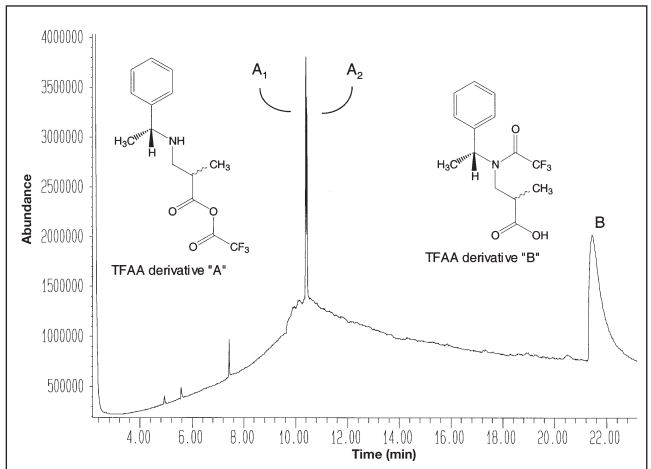

Figure 4. TIC of TFA derivatives of β-(α-methylbenzyl) amino isobutyric acid using a capillary DB-Wax. Note: partial resolution (R_S = 0.78).

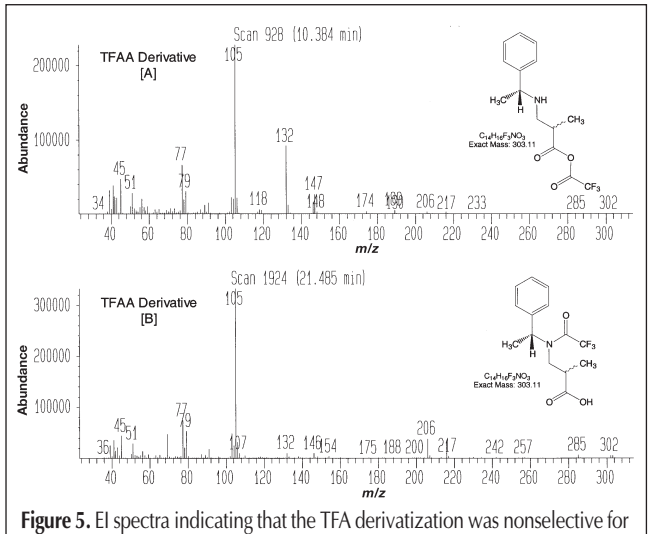

Figure 5. EI spectra indicating that the TFA derivatization was nonselective for either the amine or carboxycylic functional groups of β-(α-methylbenzyl) amino isobutyric acid.

was selected for use as the derivatizing agent for GC development because it is selective for primary (1°) and secondary (2°) amines, forming stable trifluoroacetyl (TFA) derivatives (15). TFA derivatives allowed the use of a range of stationary phases with various degrees of polarity without concern for potential damage to the columns being used. The stability of the TFA derivatives allowed for the removal of excess reagents prior to assay, preventing the reagents from being injected onto the column and damaging the stationary phase. In addition, the TFA groups had greater dipole character than TMS, producing more significant electonic changes in the TFA-derivatized molecules than their silyl analogs. The larger induced dipole for the TFA derivatives increased the opportunities for separation.

A nonpolar DB-1 column was used for preliminary experiments using TFA derivatives. The chromatography indicated good overall peak shape, but the DB-1 did not resolve the diastereomers. Nonpolar stationary phases, such as DB-1, separate primarily by boiling points (dispersive forces). Polar phases, such as DB-Wax (polyethylene glycol based), rely on interactions such as hydrogen bonding and dipole interactions between functional groups of the analyte and the stationary phase, thereby increasing selectivity. The addition of the electronegative TFA groups provided potential sites for dipole interactions between the analyte and the column stationary phase, which was a consideration when using the DB-Wax. Results of the GC–MS analysis of TFA derivatives of β-(α-methylbenzyl) amino isobutyric acid performed using a DB-Wax are shown in Figure 4.

The TFAA derivatization was found to be nonselective at a single position. EI spectra (Figure 5) indicated that monotrifluoroacetyl derivatives (*m/z* 303) were formed at both the amine and carboxylic acid. Both have very distinctive fragmentation patterns. It was also noted that partial resolution was achieved (R_S = 0.78) of monotrifluoroacetyl derivatives of the (*RR*) and (*RS*) diastereomers in which the TFA was located on the carboxyl group (16). The order of elution between the TFA derivatives of the amine and acid groups was determined by noting that only peaks (A_1 and A_2) had a fragment present at *m/z* 189. This indicated the loss of $–CO_2CF_3$, which was possible only if the TFA was on the carboxyl group. The more abundant fragments at *m/z* 147 and 132 could only occur if $–CF_3$ was on the acid site, and not if TFA had reacted with the amine. Only peak (B) had fragments at *m/z* 217 and 206; both are exclusive to the TFA amine derivative. The TFA derivatives of the (*RR*) and (*RS*) analogs were not resolved by the DB-Wax. Formation of bis-trifluoroacetyl derivatives were possible as well, but not detected in these experiments.

In an effort to improve peak shape of the TFA amine derivative while maintaining separation, a DB-210 column (50% trifluoropropyl) was used. The moderate dipole character and weak hydrogen-bonding properties of the trifluoropropyl phase were considered appropriate for interaction with the acetyl and carboxyl carbonyl groups of the TFA derivatives. Figure 6 shows the separation of TFAA derivatives of a mixture of (*RR*) and (*RS*) β-(α-methylbenzyl) amino isobutyric acid using the DB-210 column. Although excellent peak shapes resulted, it was noted that the derivatization was not specific to the amine. Furthermore, the chromatogram indicated a mixture of monotrifluoroacetyl derivatives of both the amine and acid sites, with the trifluoroacetylamine being the major product. The possibility that a bis-trifluoroacetyl derivative existed, however none was confirmed by MS. Significant resolution (R_S = 1.0) of the (*RR*) and (*RS*) diastereomers of monotrifluoroacetyl derivatives of both the acid and amine functional groups was noted. However, efforts to optimize the derivatization procedure to quantitatively form trifluoroacetylamine or bis-trifluoroacetyl derivatives were not pursued.

HPLC assay development

A number of different options were explored during development of the separation using HPLC. Preliminary experiments were designed to study solvent selectivity. The first column used was a Zorbax SB-Phenyl, chosen for its potential selectivity towards aromatic compounds. Two gradient elution runs were made using 25mM phosphate buffer adjusted to pH 3 as the weak eluent. One gradient used 10% (v/v) ACN and the other 10% (v/v) THF as the strong eluents. A solution containing a mixture of authentic diastereomers was assayed using both the ACN and THF systems. Although peak retention times (t_R) were reasonable for each gradient system (ACN t_R = 9.0 min, THF t_R = 4.5 min) no resolution of the diastereomers was observed in either case.

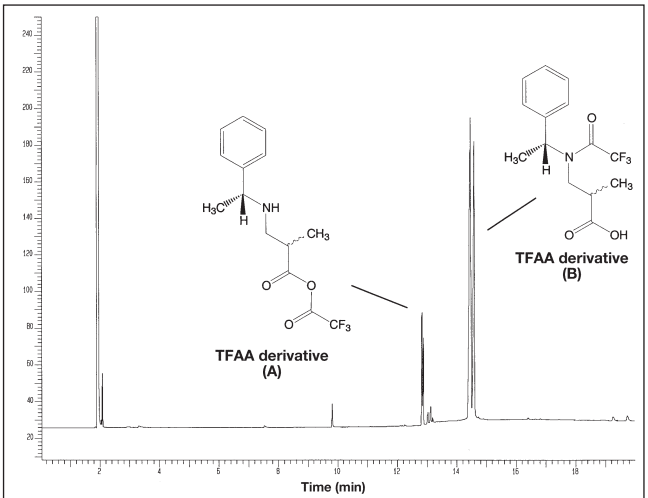

Figure 6. Resolution of TFA derivatives of β-(α-methylbenzyl) amino isobutyric acid using a DB-210.

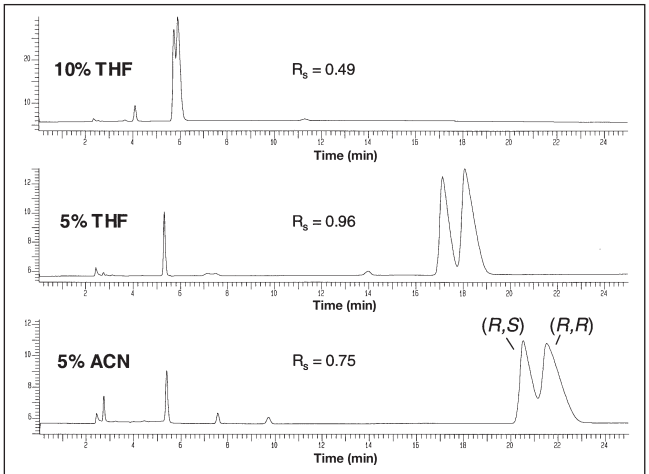

Figure 7. Comparison of solvent strength. (A) 10% THF isocratic elution, (B) 5% THF isocratic elution, and (C) 5% ACN isocratic elution. SB-Phenyl column and 25mM phosphate buffer pH 3.

In addition to the gradients, isocratic conditions were also evaluated for both THF and ACN. Partial separation of the diastereomers was observed when using 10% THF. Decreasing the THF content to 5% isocratic resulted in better resolution (R_S = 0.96 vs. 0.49). Although peak separation was improved, the peaks tailed badly. For comparison, an isocratic run at 5% ACN was also made while all other variables remained the same. It can be seen in Figure 7 that the 5% THF yielded faster assay times and greater selectivity than the 5% ACN ($R_{S\ (THF)}$ = 0.96 vs. $R_{S\ (ACN)}$ = 0.75).

Efforts were made to improve selectivity and peak tailing by adjusting the pH of the mobile phase. Because the compound is amphoteric (pK_a ~ 5 and 9), changes in the pH affected both the acid and amine functionalities (17). The pH was varied between 7.5 and 2.0. As seen in Figure 8, both overall retention and peak resolution were greatly influenced by pH changes. A slight improvement in separation at pH 2 versus pH 3 was observed, but the peaks were still tailing in both cases.

Triethylamine (TEA) (0.05% v/v) was added to the mobile phase in an attempt to minimize peak tailing. The TEA was used to help eliminate interactions between the analyte and free silanol groups on the stationary phase. A comparison between mobile phases prepared with and without TEA was performed. Although a slight improvement in separation (R_S = 1.20 vs. 1.07) was observed, peak tailing was not improved. The effect of TEA was noted to be insignificant.

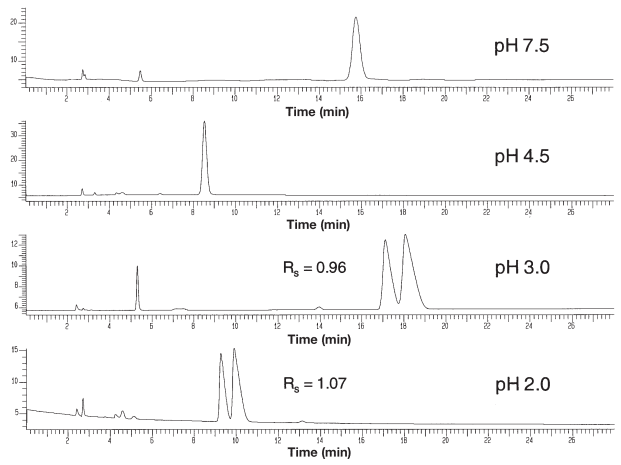

Figure 8. Effect of pH on separation and retention. (A) pH 2.0, (B) pH 3.0, (C) pH 4.5, and (D) pH 7.5. SB-Phenyl column, 5% THF–25mM phosphate buffer isocratic.

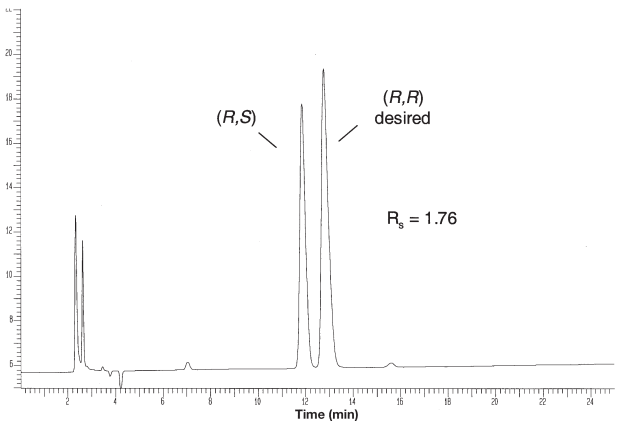

Figure 9. Final isocratic IPC assay conditions for determination of diastereomers of β-(α-methylbenzyl)amino isobutyric acid: Zorbax SB-C_8, 5% THF–5mM PSA in 25mM Na_2HPO_4 at pH 2.0 at 1 mL/min.

In addition to the study of eluent effects, a comparison of stationary phases was performed. Samples were assayed using Zorbax SB-Phenyl and Zorbax SB-C_8 columns. As mentioned previously, the SB-Phenyl was originally used to take advantage of its potential selectivity for aromatic compounds. However, it was subsequently determined that the SB-C_8 column had slightly better selectivity as indicated by a small improvement in separation ($R_{S\ (SB\text{-}Phenyl)}$ = 1.07 vs. $R_{S\ (SB\text{-}C8)}$ = 1.32).

An attempt was made to take advantage of the ionizable amine through the use of IPC (18). The addition of an ionizable alkyl sulfonate salt to the eluent was investigated as a way to improve selectivity and peak shapes by interaction with the amine. At pH 2, the amine of β-(α-methylbenzyl) amino isobutyric acid was expected to be in the cationic state, and PSA sodium salt, added to the eluent, would disassociate to form an anionic counter ion. Suppression of ionization of both the carboxylic acid group on the β-(α-methylbenzyl) amino isobutyric acid and free silanols on the column would occur at pH 2, minimizing their interaction and improving peak shape. The Zorbax SB-C_8 was suitable for interaction with the pentane side chain of the ion-pairing agent.

PSA at 2.5mM was added to 25mM sodium phosphate dibasic buffer adjusted to pH 2.0 to act as an ion-pair agent. Isocratic elution was performed using 10% THF–2.5mM PSA + phosphate buffer on the Zorbax SB-C_8. Initial chromatography indicated that the addition of PSA resulted in baseline resolution (R_S = 1.91) with nearly symmetric peak shape. Subsequent work indicated that there was some drift in t_R between runs of approximately 2.5 min with the ion-pair reagent at 2.5mM. The drifting retention times were believed to be caused by the ion-pair agent not reaching sufficient equilibrium on the column between runs, possibly as a result of incomplete saturation of the column. The concentration of PSA was increased to 5.0mM to provide an excess of counter ions on the column. A series of replicate injections over a 4-h period using the 5.0mM PSA eluent indicated no drift in retention. Increasing the concentration of PSA did, however, result in decreased retention for both peaks (~ 2.5 min), with a slight loss of resolution (R_S = 1.2). To compensate for the reduced retention, the solvent strength was reduced by lowering the percentage of THF used to 5%. This reduction in solvent strength significantly improved overall retention while still maintaining good selectivity (R_S = 1.76). These conditions were ultimately used successfully to determine %de for samples of reaction products generated during synthetic development. A chromatogram using these final assay conditions, which provided baseline resolution and relatively quick analysis times, is shown in Figure 9. The elution order was assigned by comparison against an authentic sample of the (*RR*) diastereomer as determined via ^{1}H NMR.

Conclusion

An assay for the separation of the diastereomers of β-(α-methylbenzyl)amino isobutyric acid was required. To speed assay

development, both GC and HPLC techniques were investigated concurrently. Direct assay of unmodified compound was unsuccessful by either technique. It was felt that the intra- and intermolecular interaction of the amine functional group of this amphoteric molecule was problematic. Forming derivatives, specifically at the amine, neutralized these interactions. GC development appeared very encouraging when using TFA derivatives of the amine. However, the derivatization technique originally developed for use by GC was determined to be nonselective. It was determined by GC–MS that derivatization was occurring at both the amine and carboxyl acid groups. Attempts to optimize the derivatization procedure by pushing it to selectively form trifluoroacetylamine or bis-trifluoracetyl derivatives were not pursued. LC development paralleled GC assay development by attempting to neutralize adverse chromatographic effects of the amine group by IPC. While developing the IPC method, it was determined that the pH of the mobile phase and molar concentrations of the ion-pair reagent were critical for reproducibility. Ultimately, conditions using IPC with RP-HPLC were developed with reproducible chromatography and sample stability. As a result, these assay conditions enabled fast assay turnaround and effective synthetic development to continue.

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