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Journal of Chromatography A, 684 (1994) 243–249

JOURNAL OF CHROMATOGRAPHY A

# High-performance liquid chromatographic reversed-phase and normal-phase separation of diastereomeric α-ketoamide calpain inhibitors

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First received 16 June 1994; revised manuscript received 4 July 1994

**Abstract**

α-Ketoamide calpain inhibitors contain a stereochemically labile chiral center adjacent to the keto moiety, which when epimerized results in diastereomers. High-temperature $C_4$ reversed-phase HPLC methods were developed for analysis of general purity of α-ketoamide calpain inhibitors and resulted in the separation of diastereomers of the positively charged inhibitor, AK295. Normal-phase methods that employed a Nucleosil Chiral-2 column were developed for separation of diastereomers of uncharged α-ketoamides. These methods used conditions in which the keto moiety of the inhibitors was minimally affected by the mobile phase.

## 1. Introduction

Calpain, a proteolytic enzyme, is activated in brain tissue through increased levels of intracellular calcium following a stroke. Calpain inhibitors are being evaluated as therapeutics for the treatment of neurodegeneration associated with ischemic events. The synthesis and biological activity of peptidyl α-ketoamide calpain inhibitors have been described [1–4]. These inhibitors were initially analyzed by $^1$H NMR spectroscopy, which revealed that inhibitors produced using Dakin–West chemistry [1] were a mixture of stereoisomers, and that a key chiral center was subject to epimerization. Only one of the stereoisomers was active, therefore it was important to monitor lot-to-lot variations in diastereomeric ratio and the stability of the chiral center in potential dosing formulations. Application of NMR analysis for the formulations was impractical; therefore, identification of an HPLC method for the analysis of diastereomeric ratio was required.

A number of approaches to the HPLC characterization of stereoisomers exist, including the use of chiral mobile phase additives, ligand exchange, charge transfer complex formation, and a variety of chiral stationary phases [5–7]. The inhibitors of interest (Fig. 1) possess two chiral centers, the chiral center directly adjacent (α) to the keto group was the stereocenter of interest. Epimerization of this center (L configuration in the desired isomer) results in the production of a diastereomer that has been shown to be inactive in enzyme inhibition assays [2]. HPLC is one of the most powerful separation techniques; therefore, resolution of the diastereomers by HPLC was expected to be

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0021-9673/94/$07.00 
*SSDI* 0021-9673(94)00623-7

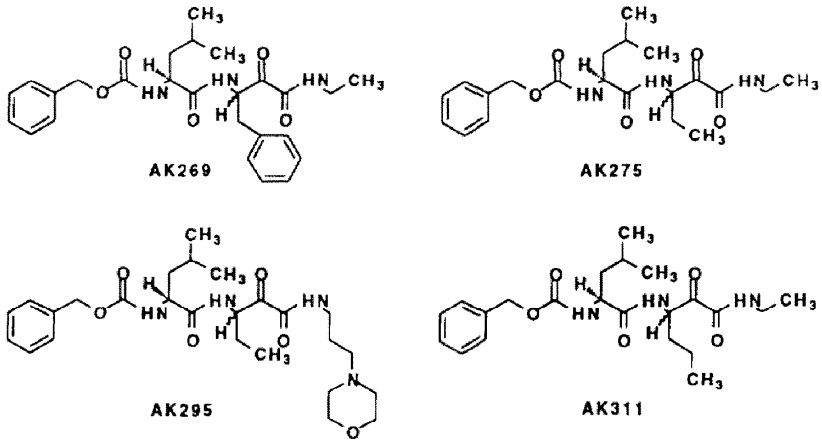


Fig. 1. Structures of calpain inhibitors. Chiral center of interest ($\alpha$ to ketone) has the hydrogen attached through a wavy bond.

relatively straightforward. However, the reactivity of the keto group present in the inhibitors was found to be a complicating factor. This paper describes the development of methods for such a separation.

## 2. Experimental

### 2.1. Materials

HPLC-grade acetonitrile, *n*-hexane, 1,4-dioxane, isopropanol and trifluoroacetic acid were obtained from Fisher Scientific. The calpain inhibitors were synthesized at Alkermes according to synthetic methods described in Refs. [1] and [2].

### 2.2. HPLC analysis

*HPLC method A (general reversed-phase system)*

The HPLC system consisted of a Hewlett-Packard 1050 HPLC system comprised of four reservoirs (only two used), a UV variable-wavelength absorbance detector, an autosampler equipped with a 100-$\mu$l loop and a HP 3396 series II integrator, and a Timberline Instruments column heater. The detector was set at 210 nm. The column used was a Vydac Protein $C_4$ column (5 $\mu$m particle size, 300 Å average pore diameter, 250 mm × 4.6 mm). The mobile phase consisted of a linear gradient system of 25% eluent B to 35% eluent B over 40 min (eluent A: 0.1% trifluoroacetic acid in water; eluent B: acetonitrile). The flow-rate was 1 ml/min. The column temperature was varied, with 60°C found to be the best temperature examined.

*HPLC method B (reversed-phase system for AK295)*

The HPLC system consisted of a Hewlett-Packard 1090M system Series II. The detector was set at 210 nm. The column used was a Vydac Protein $C_4$ column (5 $\mu$m particle size, 300 Å average pore diameter, 250 mm × 4.6 mm). The mobile phase consisted of a linear gradient system of 15% eluent B to 35% eluent B over 40 min (eluent A: 0.1% trifluoroacetic acid in water; eluent B: acetonitrile). The flow-rate was 1 ml/min. The column temperature was 60°C.

*HPLC method C (Cyclobond column for analysis of AK269)*

The HPLC system consisted of a Hewlett-Packard 1090M system. The detector was set at 210 nm. The column used as an ASTEC Cyclobond I ($\beta$-cyclodextrin) column. The mobile

phase consisted of a linear gradient from 27.5% eluent B to 37.5% eluent B over 30 min (eluent A: 0.1% triethylammonium phosphate, pH 4.0; eluent B: MeOH). The flow-rate was 1 ml/min. The column temperature was kept at 0°C by immersing the column in an ice bath.

*HPLC method D (normal-phase analysis of AK275 on a Phenomenex column)*

The HPLC system consisted of a Hewlett-Packard 1050 HPLC system comprised of four reservoirs (only one used), a UV variable-wavelength absorbance detector, an autosampler equipped with a 100-$\mu$l loop, and a HP 3396 series II integrator. The detector was set at 210 nm. The column was a Phenomenex Chirex (*S*)-Leu, (*R*)-1-($\alpha$-naphthyl)ethylamine (NEA) column (250 mm × 4.6 mm). According to the manufacturer, this column "consists of an optically pure amino acid derivative covalently bonded to $\delta$-aminopropyl-silanized silica gel". The mobile phase consisted of an isocratic system of dioxane–hexane–acetonitrile (17:78:5). The flow-rate was 1 ml/min. The column temperature was ambient.

*HPLC method E (normal-phase analysis of AK275 on a Nucleosil column)*

The HPLC system consisted of a Hewlett-Packard 1050 series HPLC system comprised of four reservoirs (only one used), a UV variable-wavelength absorbance detector, an autosampler equipped with a 100-$\mu$l loop, and a HP 3396 series II integrator. The detector was set at 210 nm. The column was a Nucleosil Chiral-2 column (250 mm × 4 mm; Macherey–Nagel). The column has three chiral centers resulting from the bonding of L-tartaric acid and L-nitrobenzyl-phenylethylamine to Nucleosil silica via a propyl spacer. The mobile phase consisted of an isocratic system of hexane–1,4-dioxane–acetonitrile (86:11:3, v/v/v). The flow-rate was 1 ml/min. A new Nucleosil Chiral-2 column has to be preconditioned before use by washing the column with 30 ml methylene chloride, 30 ml isopropanol and 30 ml hexane at a flow-rate of 1 ml/min. The column temperature was ambient.

*HPLC method F (normal-phase analysis of AK311 on a Nucleosil column)*

The system is identical to method E except the mobile phase consisted of an isocratic system

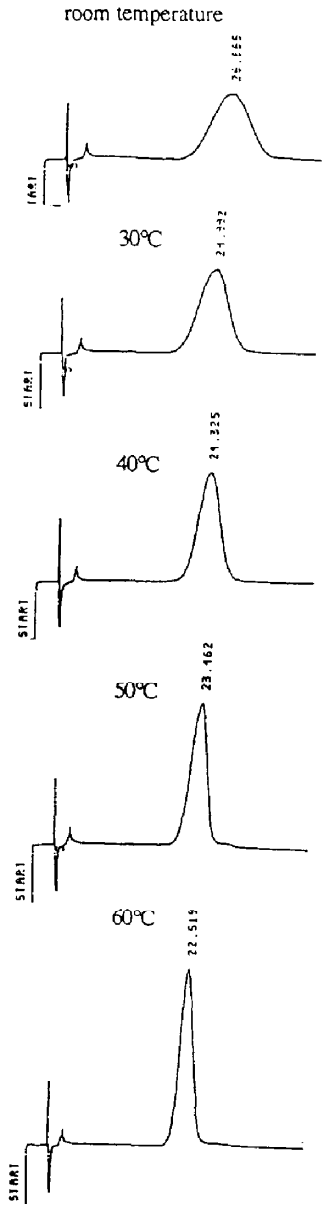


Fig. 2. Variable-temperature analysis (HPLC method A, reversed-phase) of AK275. Values at peaks are retention times in min.

of *n*-hexane–1,4-dioxane–acetonitrile (87:10:3, v/v/v).

*HPLC method G (normal-phase analysis of AK269 on a Nucleosil column)*

The system is identical to method E except the mobile phase consisted of an isocratic system of *n*-hexane–isopropanol–acetonitrile (94:4:2, v/v/v).

## 3. Results and discussion

The structures of the calpain inhibitors studied are shown in Fig. 1. The diastereomeric ratio of the calpain inhibitors could be evaluated by NMR techniques; however, this was time consuming and required significant sample quantity. Gas chromatography techniques were not an option, as the inhibitors were not sufficiently volatile. Therefore, an HPLC method for determining the diastereomeric ratio of inhibitor was needed.

A reversed-phase system (HPLC method A) was used for initial HPLC analyses. The effect of column temperature can be seen in Fig. 2. In the chromatograms of AK275 run near room temperature, the peak was broad. Increasing the column temperature improved the peak shape, increased the efficiency of the column and decreased the pressure drop. The best conditions were observed using a column temperature of 60°C. While high-temperature reversed-phase HPLC analysis has proven useful for general purity analysis, diastereomeric resolution was seen only for positively charged AK295 (Fig. 3, HPLC method B). Unfortunately, such high-temperature reversed-phase methods did not lead to separation of the diastereomers of the neutral compounds, AK275, AK311 and AK269.

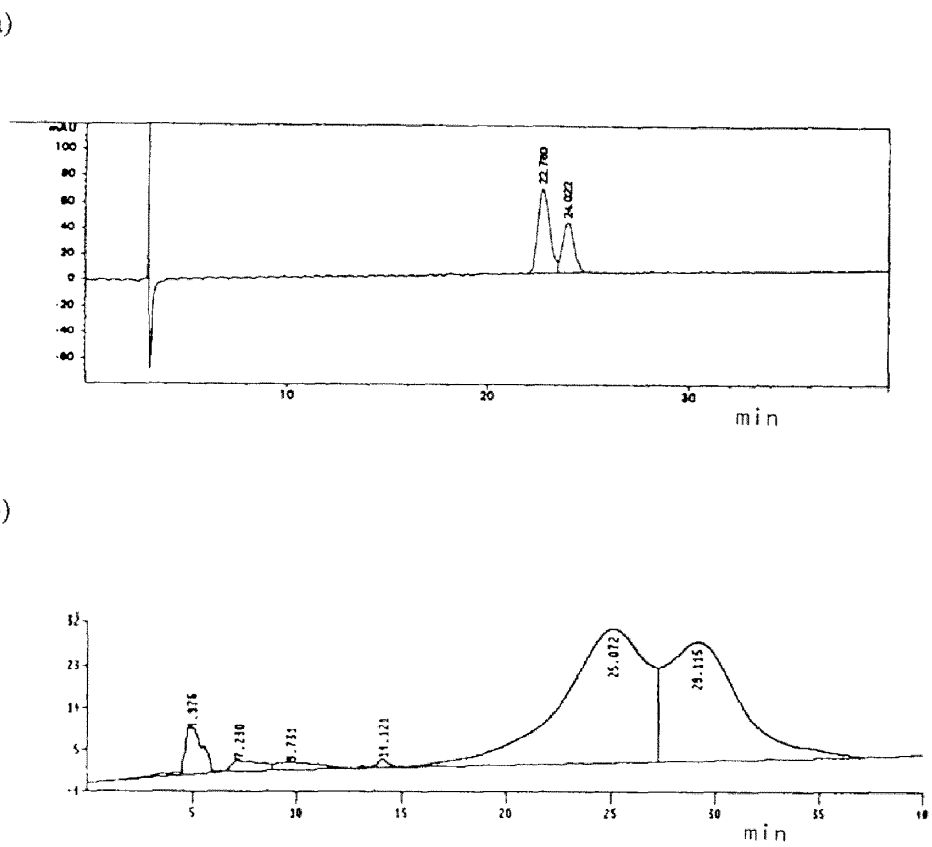


Fig. 3. Reversed-phase separation of calpain inhibitors: (a) AK295 (method B) and (b) AK269 (method C).

A large number of other aqueous-based HPLC systems were examined including Chiral Triacel, Chiralcel OD-R, cyclodextrin-based columns (Cyclobond I, II and III) and hydrophilic interaction (POROS OH/H from PerSeptive Biosystems). A system using Cyclobond II (HPLC method C) was found to give marginal separation of the diastereomers of AK269 (Fig. 3), which has the requisite aromatic group adjacent to the chiral center of interest. However, no separation of the diastereomers of AK275 and AK311 was seen in any reversed-phase system.

NMR studies indicated that the peak broadening seen in the chromatographic systems employing aqueous phases might have resulted from the reaction of the keto group of the inhibitors with water in mobile phase to form the hydrate. This ketone–hydrate equilibrium was affected by temperature, with the equilibrium shifted toward the ketone at higher temperatures (Fig. 4). Such a shift in the equilibrium might partially account for the improved peak shape in the high-temperature reversed-phase chromatography seen in Fig. 2. As the inclusion of water in the eluents appeared to be problematic, normal-phase HPLC methods were examined.

The neutral calpain inhibitors used in these studies were very hydrophobic molecules, which made them compatible with normal-phase HPLC analysis. Most of the inhibitors absorb strongly at 210 nm; therefore, we wanted to use 210 nm as the detection wavelength for maximum sensitivity. Unfortunately, the use of 210 nm limits the number of solvents that can be considered for the mobile phase. The solvents that were evaluated included *n*-hexane, *n*-heptane, dioxane, 1,2-dichloroethane, tetrahydrofuran and a few alcohols. Isocratic elution systems were used, which allowed the use of solvents such as 1,2-dichloroethane, which has a UV cut-off of 228 nm. The mobile phases containing hexane–dioxane or hexane–isopropanol mixtures were found to have the greatest promise for the diastereomeric separation. The addition of a small amount of acetonitrile sharpened the peaks and improved resolution.

The first observed chromatographic separation of the diastereomers of AK275 was performed

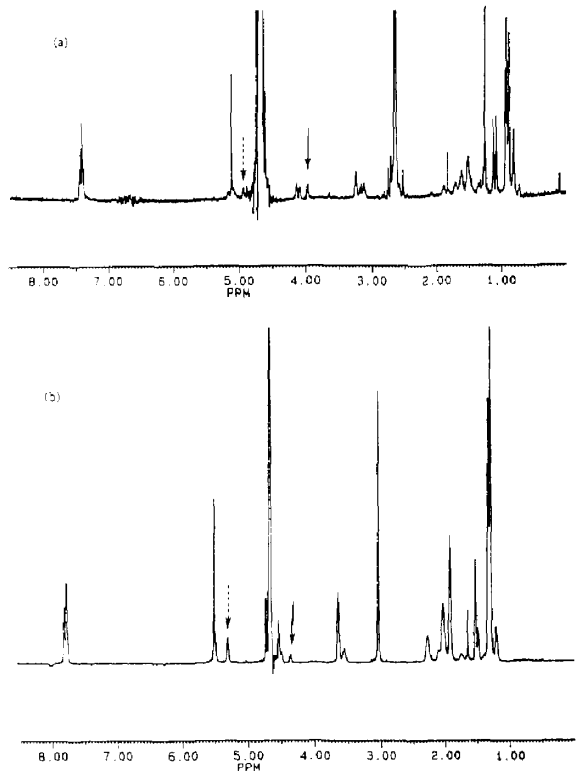


Fig. 4. Variable temperature $^1$H NMR analysis of AK275 in $^2H_2O$–[$^2H_6$]dimethyl sulfoxide (60:40): (a) room temperature; (b) 60°C. The dashed arrow indicates the position of the resonance of the hydrogen $\alpha$ to ketone. The solid arrow indicates the position of the resonance of the hydrogen $\alpha$ to the hydrated form of the ketone.

using a Phenomenex Chirex (*S*)-Leu and (*R*)-NEA column (Fig. 5a, method D). The mobile phase employed was hexane–1,4-dioxane–acetonitrile. It was subsequently found that a Nucleosil Chiral-2 column from Macherey–Nagel also resulted in separation of the diastereomers of AK275 (Fig. 5b, method E). New Nucleosil Chiral-2 columns had to be pretreated with methylene chloride, isopropanol and hexane before use, as the new column appeared to contain some storage solution with a high absorbance response at 210 nm. The inhibitor, AK311, was also successfully separated on a Chiral-2 column with a mobile phase of hexane–1,4-dioxane–acetonitrile (Fig. 5c, method F). AK269 required the use of a slightly different solvent system (hexane–isopropanol–acetoni-

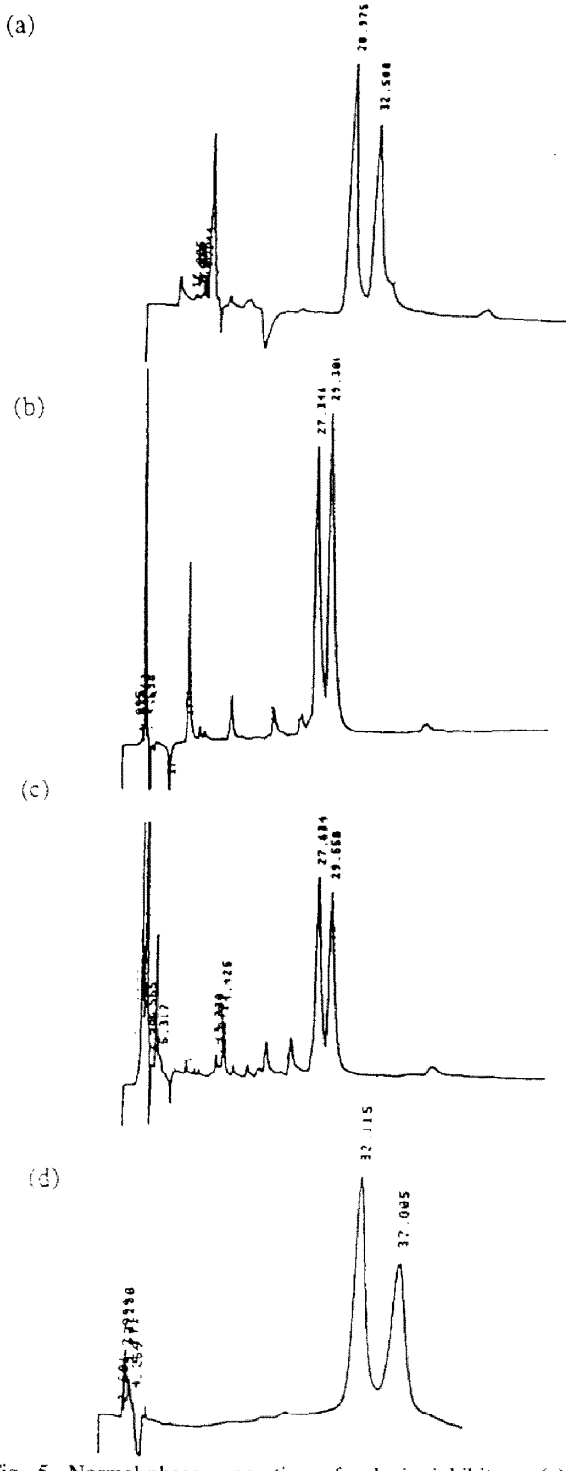


Fig. 5. Normal-phase separation of calpain inhibitors: (a) AK275 (method D), (b) AK275 (method E), (c) AK311 (method F) and (d) AK269 (method G).

trile, method G) with the Chiral-2 column for resolution of the diastereomers (Fig. 5d). While the isopropanol used in method G could potentially react with the keto group of AK269 to form hemi-ketals, previous $^1$H NMR studies using methanol revealed that the rate of formation of such ketals at room temperature was slow relative to the rate of formation of a hydrate with water [2]. The isocratic elution system employed in the normal-phase methods minimized the background noise, allowing for detection at 210 nm. The relative and absolute retention times of the diastereomer peaks could be adjusted by small changes of the composition of mobile phase. Such adjustments have been required for analysis of diastereomeric ratio in various formulations (data not shown). Normal-phase separation of diastereomers of AK295 was not examined, since adequate separation was seen on the reversed-phase system (method B).

## 6. Conclusions

The high-temperature $C_4$ reversed-phase HPLC system could be used for analysis of general purity of the calpain inhibitors and allowed the separation of diastereomers of the positively charged inhibitor, AK295. This type of HPLC system has been used for supporting synthetic work, formulation evaluation, and pharmacokinetic studies. The normal-phase Nucleosil Chiral-2 stationary phase has been employed in the studies of epimerization rates and in formulation evaluation. The normal-phase methods have also been used for general purity assessment as a complement to the reversed-phase method. The two general methods (high-temperature reversed-phase analysis and normal-phase chiral analysis) that were found to be most useful for the analyses of calpain inhibitors were used in a manner in which the keto moiety of the inhibitors was minimally affected by the mobile phase. It is clearly important to take molecular reactivity into consideration when developing analytical assays.

## Acknowledgements

The authors wish to thank Dr. Scott L. Harbeson and Robert Barrett for the syntheses of the inhibitors used in this study.

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