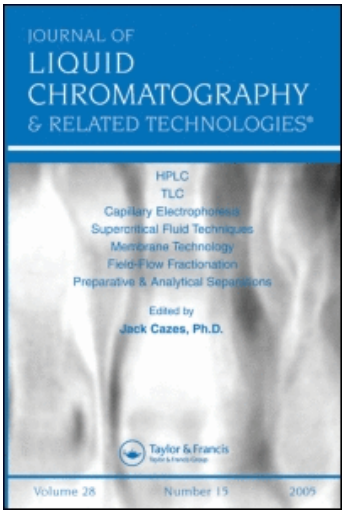

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SEPARATION OF ISOMERS OF *CIS*- AND *TRANS*-4-AMINOCYCLOHEXYLACETIC ACID AND CORRESPONDING ETHYL ESTER

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ABSTRACT

Synthesis of PD 158771 (Figure 1) involved 4-amino-cyclohexylacetic acid and its ethyl ester intermediates which required purification to obtain the desired corresponding *trans*-isomer. HPLC methods were developed for the separation of *cis*- and *trans*-4-aminocyclohexylacetic acid and the corresponding ethyl esters following pre-column derivatization with *N*α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent). The chromatographic conditions were varied to achieve optimal separation.

INTRODUCTION

PD 158771 has been proposed as a central nervous system (CNS) agent particularly useful as an anxiolytic agent and for possible treatment of schizophrenia.[1]

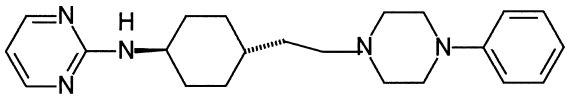

Figure 1. Structure of PD 158771.

The desired form of PD 158771 is the *trans*-isomer. To avoid significant amount of the *cis*-isomer present as an undesired impurity in the final product, purification of the *trans*-4-aminocyclohexylacetic acid intermediate is important during process development. For a typical sample of 4-aminocyclohexylacetic acid, the undesired *cis*-isomer ranges from a few percent up to 30 percent or higher. The sample was selectively enriched using hydrogen chloride in ethanol to obtain pure *trans*-4-aminocyclohexylacetic acid. This compound was then esterified to produce *trans*-4-aminocyclohexylacetic acid ethyl ester. To ensure purity of these important intermediates, good analytical methods are required.

Marfey's reagent has been successfully used to react with the optical isomers of amino acids[2-19] and amines[20] to form diastereomeric N-aryl derivatives which can be separated by conventional HPLC methods. The derivatives have an absorption maximum at 340 nm with an extinction coefficient of $3x10^4$ and therefore they can be detected by UV with high sensitivity.[3]

The present work describes the application of Marfey's reagent to facilitate HPLC separation of the *cis*- and *trans*-isomers of 4-aminocyclohexylacetic acid and corresponding ethyl esters (Figure 2). The *trans*-isomers are two important intermediates to make PD 158771.

EXPERIMENTAL

Equipment

The HPLC system consisted of a Hitachi 6200A intelligent pump, a Micromeritics 728 autosampler, a Rheodyne 7010 injection valve with a 20 μL loop, a Hitachi L-4000 variable wavelength UV detector, a Waters 410 Differential Refractometer equipped with a column oven, and a Hitachi D-2500 Chromato-integrator. The HPLC columns were a Hypersil BDS C18, 250x4.6 mm, 5 μm in particle size which was obtained from Alltech Associates (Deerfield, IL), and a YMC-AQ C18, 250x4.6mm, 5 μm in particle size which was purchased from YMC (Wilmington, NC).

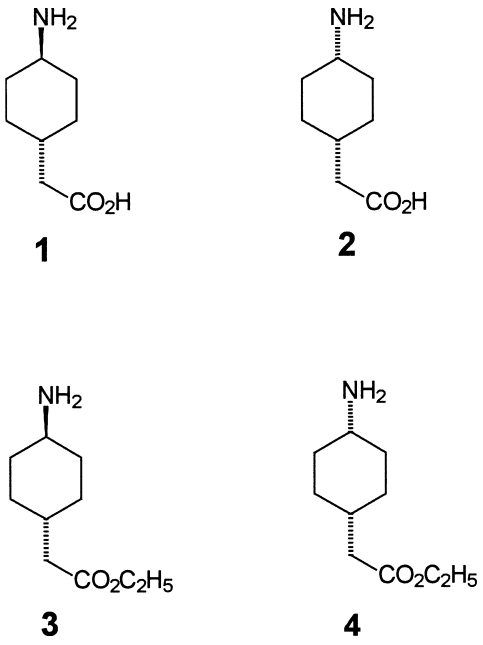

Figure 2. Structures of (**1**) *trans*-4-aminocyclohexylacetic acid, (**2**) *cis*-4-aminocyclohexylacetic acid, (**3**) *trans*-4-aminocyclohexylacetic acid ethyl ester, and (**4**) *cis*-4-aminocyclohexylacetic acid ethyl ester.

Chemicals

*N*α-(2,4-Dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent) was purchased from Aldrich Chemical Company (Milwaukee, WI). Acetonitrile (CH_3CN, HPLC grade), methanol (CH_3OH, HPLC grade), sodium bicarbonate ($NaHCO_3$), ammonium dihydrogen phosphate ($NH_4H_2PO_4$), hydrochloric acid (HCl), and phosphoric acid (H_3PO_4) were obtained from EM Science (Gibbstown, NJ). Triethylamine (TEA) was obtained from Burdick & Jackson (Muskegon, MI). 4-Aminocyclohexylacetic acid and its ethyl ester were synthesized at the Chemical Development Department, Parke-Davis Pharmaceutical Research Division (Holland, MI).[21] Water was obtained from a NANOpure ultrapure water system, Barnstead/Thermolyne (Dubuque, IA).

Chromatographic Conditions

For underivatized 4-aminocyclohexylacetic acid and ethyl ester, the mobile phase was 15 CH_3OH/85 0.05M $NH_4H_2PO_4$ + 6mL TEA/L (pH 7.0/H_3PO_4).

The flow was 1.0 mL/min, the column temperature was 30°C, and the detection device was a refractive index detector. For derivatized 4-aminocyclohexylacetic acid and ethyl ester, the mobile phase was CH_3CN/0.05M TEA (pH3.0/H_3PO_4), and the volume ratio was varied depending upon the sample. The flow rate was 2.0 mL/min, the column temperature was at room temperature, and the detection device was UV at 340 nm. The injection volume was 20 μL.

Derivatization Procedure

The following solutions were first prepared: 1.5 to 2 mg/mL of sample in acetonitrile/water (1/5, v/v), 5 mg/mL of *N*α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent) in acetonitrile, 1 M aqueous sodium bicarbonate, and 1 N aqueous hydrochloric acid. The derivatization was carried out by mixing 0.25 mL of sample solution, 0.25 mL of Marfey's reagent, and 0.03 mL sodium bicarbonate solution in a sealed reaction vial. The vial was then heated at 40°C with stirring for about 30 minutes. 0.03 mL of 1 N hydrochloric acid solution was added to the vial and mixed well. The content of the vial was diluted to 2.0 mL with mobile phase, and the solution was ready to be injected. The amount of reagents used can be varied proportionally according to the amount of sample to be derivatized.

RESULTS AND DISCUSSION

Direct HPLC Separation

Direct separation of *cis*- and *trans*-isomers of 4-aminocyclohexylacetic acid and ethyl esters using a refractive index detector was our desired goal at an early stage of process development. Attempts using various C18, C8, CN columns, and the ion-pairing principle on C18 and C8 columns to separate *cis*- and *trans*-isomers of 4-aminocyclohexylacetic acid did not give satisfactory results.

The best separation obtained for *cis*- and *trans*-isomers of 4-aminocyclohexylacetic acid ethyl ester is shown in Figure 3A. The isomer peaks were broad and not baseline resolved. When the sample was purified further, the amount of *cis*-isomer is expected to be lower. The *cis*-isomer peak was completely buried in the front end of *trans*-isomer peak and this made the separation of *cis*-isomer from *trans*-isomer impossible as seen in Figure 3B. Attempts to separate *cis*- and *trans*-isomers of 4-aminocyclohexylacetic acid along this approach were also unsuccessful. Alternative methods were needed.

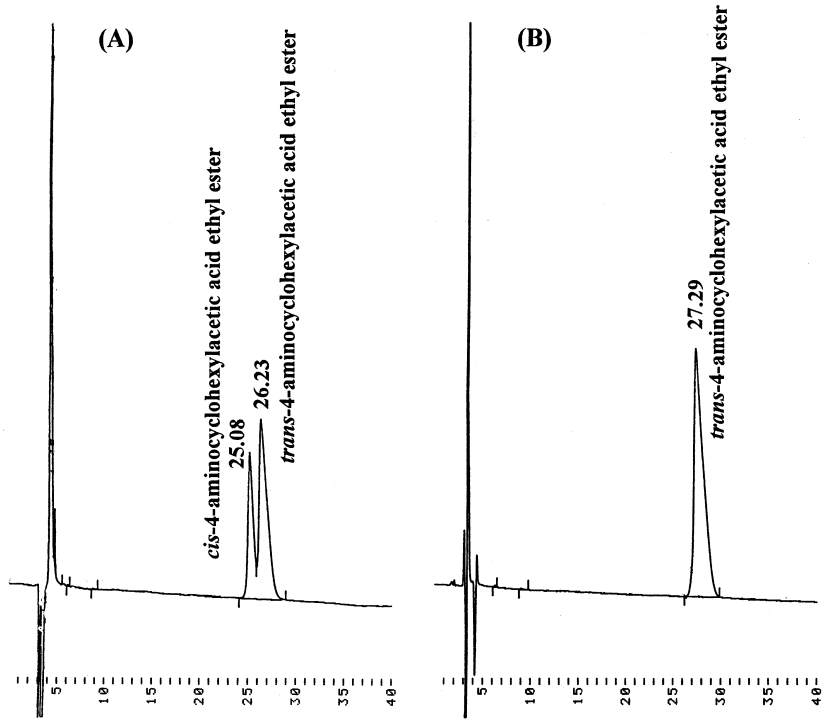

Figure 3. Separation of *cis*- and *trans*-4-aminocyclohexylacetic acid ethyl ester. (A) a mixture of 35 % *cis*-isomer and 65 % *trans*-isomer, (B) a mixture of 0.2 % *cis*-isomer and 99.8 % *trans*-isomer. Column: YMC-AQ, C18, 5 μm, 250x4.6 mm; Mobile phase: 15 CH_3OH/85 0.05M $NH_4H_2PO_4$ + 6mL TEA/L (pH7.0/H_3PO_4); Flow: 1.0 mL/min.; Column temperature: 30 °C; Sample amount injected: ~100 μg.

Derivatization

*N*α-(2,4-Dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent) is a chiral compound and was initially developed for separation of optical isomers of amino acids. Subsequently, it has been widely employed as a derivatizing reagent for chiral separation of amino acids as diastereomers by conventional HPLC methods.

Although our work did not involve the separation of optical isomers, we found that this reagent is also very useful as a derivatizing reagent for the *cis*- and *trans*-isomers of 4-aminocyclohexylacetic acid and ethyl esters.

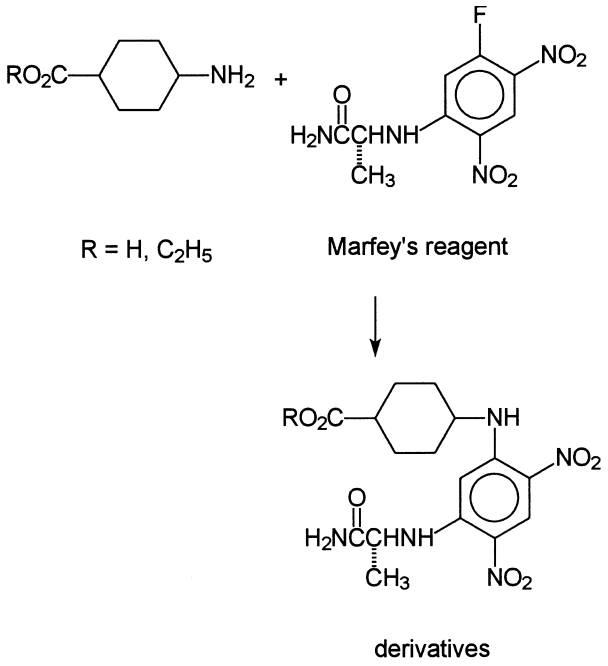

Figure 4. Scheme for derivatization reaction.

The resulting isomers can easily be separated. Additionally, the derivatized compounds give a very intense absorption at the wavelength of about 340 nm, and made the use of UV detection desirable. The derivatization chemistry is shown in Figure 4.

A sample contained *trans*-4-aminocyclohexylacetic acid ethyl ester as a major component while the other three isomers present as minor components was selected to study the derivatization time effect. The derivatization reactions were carried out for 30 and 60 minutes, respectively, at three sample concentrations. No significant change was observed at either 30 or 60 minutes of reaction time up to 5 mg/mL of sample concentration as seen in Table 1. It seems that the reaction was complete within 30 minutes, which is the derivatization time included in the procedure described in the Experimental section.

The effect of Marfey's reagent concentration on the same sample was studied by varying Marfey's reagent concentration from 3 mg/mL to 9 mg/mL. At 3 mg/mL, the Marfey's reagent was slightly in excess and at 9 mg/mL, the

Table 1

Effect of Derivatization Time for a Sample Containing *trans*-4-Aminocyclohexylacetic Acid Ethyl Ester as a Major Component

	3 mg/mL		4 mg/mL		5 mg/mL	
	30 min	60 min	30 min	60 min	30 min	60 min
cis-4-aminocyclohexylacetic acid	4.97%	4.88%	4.87%	4.70%	4.85%	4.74%
trans-4-aminocyclohexylacetic acid	1.91%	1.97%	1.92%	1.93%	1.95%	2.03%
cis-4-aminocyclohexylacetic acid ethyl ester	7.73%	7.34%	7.44%	7.12%	7.30%	6.85%
trans-4-aminocyclohexylacetic acid ethyl ester	85.40%	85.79%	85.77%	86.25%	85.90%	86.36%

Table 2

Effect of Marfey's Reagent Concentration for a Sample Containing *trans*-4-Aminocyclohexylacetic Acid Ethyl Ester as a Major Component

	3 mg/mL	4 mg/mL	5 mg/mL	6 mg/mL	7 mg/mL	9 mg/mL
cis-4-aminocyclohexylacetic acid	4.88%	4.70%	4.71%	4.71%	4.71%	4.74%
trans-4-aminocyclohexylacetic acid	1.97%	1.93%	1.99%	2.01%	2.01%	2.05%
cis-4-aminocyclohexylacetic acid ethyl ester	7.34%	7.12%	6.95%	6.89%	6.82%	6.69%
trans-4-aminocyclohexylacetic acid ethyl ester	85.79%	86.25%	86.34%	86.40%	86.47%	86.51%

Marfey's reagent was more than three time in excess. Again, no significant change was observed as seen in Table 2. Five mg/mL concentration of the Marfey's reagent was chosen for the described procedure in the Experimental section.

Separation of *cis*- and *trans*-4-Aminocyclohexylacetic Acid

Since the ratio of *cis*- and *trans*-isomers can vary significantly from sample to sample, the conditions were optimized to achieve a good resolution between the two isomers. As seen in Figure 5, the derivatized 4-aminocyclohexylacetic acid isomers were separated well. The *cis*-isomer was eluted earlier than the *trans*-isomer. The resolution, run time, and the peak shapes of the isomers were good.

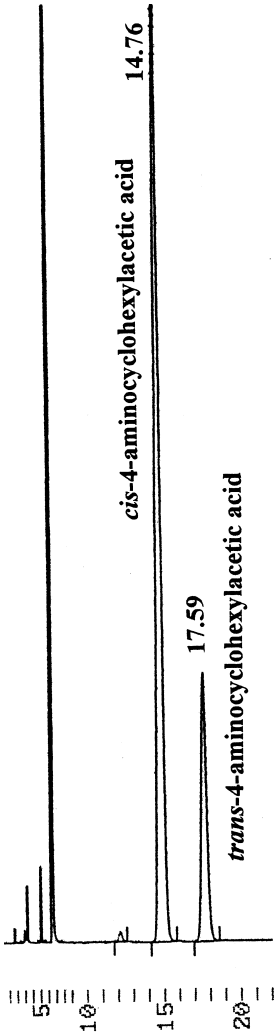

Figure 5. A typical chromatogram for separation of derivatized *cis*- and *trans*-4-aminocyclohexylacetic acid. Column: Hypersil BDS, C18, 5 μm, 250x4.6 mm; Mobile phase: 30 CH_3CN/70 0.05M TEA (pH3.0/H_3PO_4); Flow: 2.0 mL/min; UV detection: 340 nm; Injection volume: 20 μL.

Separation of *cis*- and *trans*-4-Aminocyclohexylacetic Acid Ethyl Ester

In the reaction sequence, *trans*-4-aminocyclohexylacetic acid ethyl ester was made via the esterification of *trans*-4-aminocyclohexylacetic acid. The amount of *cis*-isomer at this stage was expected to be small. As seen in Figure

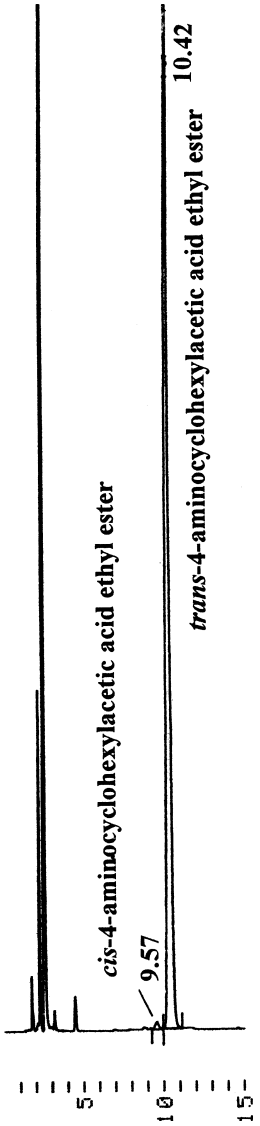

Figure 6. A typical chromatogram for separation of derivatized *cis*- and *trans*-4-aminocyclohexylacetic acid ethyl ester. Column: Hypersil BDS, C18, 5 μm, 250x4.6 mm; Mobile phase: 45 CH_3CN/55 0.05M TEA (pH3.0/H_3PO_4); Flow: 2.0 mL/min; UV detection: 340 nm; Injection volume: 20 μL.

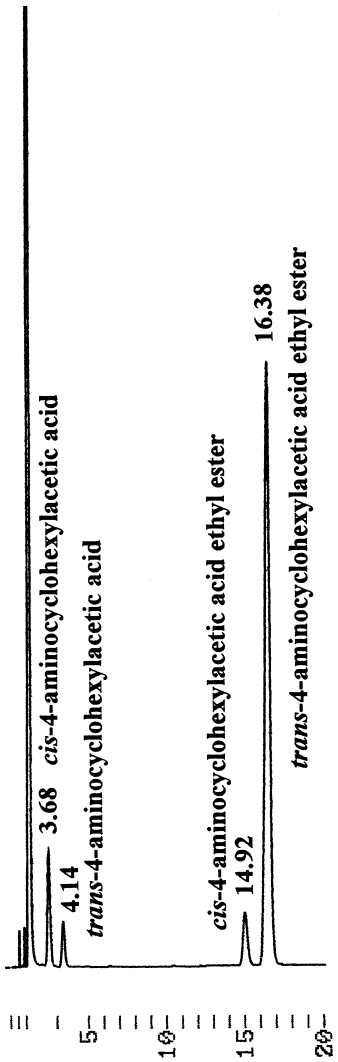

Figure 7. A typical chromatogram for simultaneous separation of derivatized *cis*- and *trans*-4-aminocyclohexylacetic acid and its ethyl ester. Column: Hypersil BDS, C18, 5 μm, 250x4.6 mm; Mobile phase: 40 CH_3CN/60 0.05M TEA (pH3.0/H_3PO_4); Flow: 2.0 mL/min; UV detection: 340 nm; Injection volume: 20 μL.

6, good separation was obtained for the derivatized 4-aminocyclohexylacetic acid ethyl ester. The *cis*-isomer was eluted earlier than the *trans*-isomer. Again, the resolution, run time, and peak shapes of the isomers were good.

Simultaneous Separation of the 4-Aminocyclohexylacetic Acids and Ethyl Esters

When the esterification reaction was not quite complete, all four compounds could potentially be present in a sample of *trans*-4-aminocyclohexylacetic acid ethyl ester. It would be very desirable to have a condition which can separate all four compounds.

The ratio of CH_3CN/0.05M TEA (pH 3.0) were varied to optimize the separation for all four compounds simultaneously. The mobile phase consisting of 40 CH_3CN/60 0.05M TEA (pH 3.0) gave good separation for all four compounds within 20 minutes run time as seen in Figure 7.

CONCLUSION

HPLC methods have been developed and optimized to successfully separate the *cis*- and *trans*-isomer pairs of both 4-aminocyclohexylacetic acid and 4-aminocyclohexylacetic acid ethyl ester following the pre-column derivatization with Marfey's reagent.

Better HPLC separation was achieved for the derivatized compounds with increased sensitivity when monitored by UV @ 340 nm. The methods developed are simple, sensitive, and allow the detection of a small amount of undesired *cis*-isomer in the presence of a large amount of desired *trans*-isomer.

These methods have been routinely used to assess the isomeric purities of the desired PD158771 intermediates, *trans*-amino-cyclohexylacetic acid and *trans*-aminocyclohexylacetic acid ethyl ester.

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