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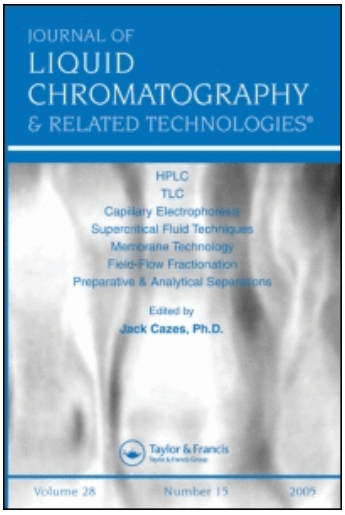

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M. M. Hefnawy[a]; J. T. Stewart[a]
[a] Department of Medicinal Chemistry, College of Pharmacy The University of Georgia, Athens, GA, USA

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HPLC SEPARATION OF METYROSINE ENANTIOMERS AS METHYL ESTERS DERIVATIZED WITH 2, 3, 4, 6-TETRA-O-ACETYL-β-D-GLUCOPYRANOSYL ISOTHIOCYANATE

Mohamed M. Hefnawy, James T. Stewart*

Department of Medicinal Chemistry
College of Pharmacy
The University of Georgia
Athens, GA 30602-2352 USA

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ABSTRACT

Enantiomeric resolution of racemic metyrosine as methyl esters was investigated following the formation of diastereomeric thioureas with 2, 3, 4, 6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (TAGIT). Baseline separation (R_s>1.5) was achieved for the derivatized enantiomers on an octadecylsilane column under isocratic conditions using a mobile phase consisting of 35:65 v/v acetonitrile-distilled water containing 0.1% triethylamine pH 4.0 (adjusted with trifluoroacetic acid) at a flow rate of 1.0 mL and detection at 250 nm. Retention times, peak resolution, and separation factors were tabulated and a possible mechanism of separation is discussed.

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INTRODUCTION

Chiral chromatography has become increasingly important in the pharmaceutical and agrochemical fields. Many therapeutic agents marketed today are racemic mixtures in spite of significant differences in pharmacological, pharmacodynamic, and pharmacokinetics of the individual isomers. Usually, one isomer is more active, toxic, or inactive than the other.[1-4] New FDA regulatory guidelines are forcing the pharmaceutical industry to re-evaluate the enantiomeric purity and activity/toxicity of any new drug.[5]

Metyrosine is clinically administered as a racemate for the control of hypertension in patients with phaeochromocytoma and may be administered as a pre-operative medication for those patients for whom surgery is contraindicated.[6] The L enantiomer is biologically active and the D enantiomer is inactive.[6]

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A review of the literature revealed that metyrosine has been determined in serum by high performance liquid chromatography (HPLC) in the presence of its major metabolite α-methyldopa using solid phase extraction and fluorescence detection.[7] It was also assayed in biological fluids and tissues by gas chromatography-mass spectrometry (GC-MS).[8] It has also been determined fluorometrically in biological fluids following a liquid-liquid extraction procedure.[9] Metyrosine in its dosage forms has also been determined either polarographically through treatment with nitrous acid[10] or colorimetrically via its reaction with 4-amino antipyrine in the presence of an alkaline oxidizing agent.[11] The USP 23 recommends a non-aqueous titration method with potentiometric detection of the end point for the evaluation of the bulk drug substance.[12] No HPLC methods have been reported for the separation of the metyrosine enantiomers. Successful attempts to resolve enantiomers in general by liquid chromatography using chiral derivatization reagents have been reported.[13-16] In particular, pre-column chiral derivatization methods have been developed for the resolution of enantiomeric amino acids by HPLC.[17-20] These methods usually require concomitant protection of free carboxyl residues prior to the chiral derivatization reaction.

This paper describes an HPLC procedure for the separation of L and D metyrosine enantiomers as their methyl esters followed by derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (TAGlT) to form diasteromeric thioureas of metyrosine. The method employs reversed phase chromatography on an octadecylsilane column with a run time of approximately 15 min.

EXPERIMENTAL

Reagents and Chemicals

Racemic metyrosine was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA)-L-metyrosine was donated by Fluka (Ronkonkoma, NY, USA). The derivatizing reagent TAGIT, anhydrous hydrazine and trifluoroacetic acid were purchased from Aldrich Chemical Co., (Milwaukee, WI, USA). Triethylamine was obtained from Eastman Kodak (Rochester, NY, USA). HPLC grade, absolute methanol, acetonitrile, and water were obtained from J.T. Baker (Phillipsburg, NJ, USA). 0.5% w/v TAGIT derivatizing reagent solution (5 mg/mL in acetonitrile) and 0.5% v/v hydrazine solution (0.5 mL/100 mL in acetonitrile) were prepared fresh daily.

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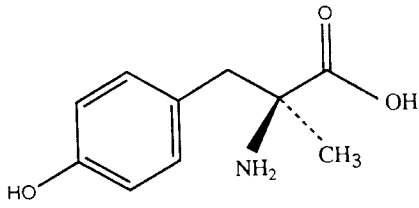
HPLC Conditions

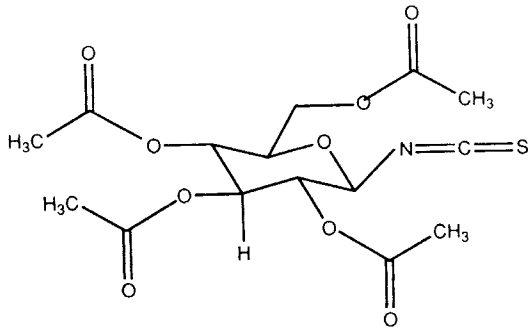
Chromatographic separation was performed using an isocratic HPLC system consisting of a Beckman Model 110A solvent delivery module (Beckman, San Ramon, CA, USA) and a Spectroflow Model 757 absorbance detector (Kratos Analytical, Ramsey, NJ, USA) set at 250 nm. Each chromatogram and peak area response were recorded on a HP Model 3290 integrator (Hewlett Packard, Avondale, PA, USA). The stationary phase was a 250 x 4.6 mm id Spherisorb ODS 5 μm column (Keystone Scientific, Inc., Bellefonte, PA, USA). The mobile phase consisted of a mixture of acetonitrile-water 35:65 v/v containing 0.1% triethylamine (pH adjusted to 4.0 with trifluoroacetic acid) and delivered at a flow rate of 1.0 mL/min at ambient temperature. The mobile phase was filtered through a 0.45 μm filter (Altech, Deerfield, IL, USA).

Derivatization Procedures

Metyrosine methyl esters were prepared by treatment of racemic metyrosine with absolute methanol and concentrated sulfuric acid.[21] Five mg of the isolated racemic metyrosine methyl esters was dissolved in 50:50 v/v acetonitrile-water containing 0.5% v/v triethylamine to give a final concentration of 1 mg/mL. A 50 μL aliquot of the metyrosine solution was transferred to a 1 mL volumetric tube and heated at 45°C for 15 min., ten μL of TAGIT reagent solution was added, and the solution heated at 45°C for an additional 2 hr.



Metyrosine



TAGIT

Figure 1. Chemical structures of metyrosine and TAGIT derivatizing reagent.

Then 10 μL of the hydrazine solution was added and the mixture heated at 45°C for 15 min. After evaporation of the mixture under a nitrogen stream at ambient temperature, the residue was reconstituted in 250 μL of mobile phase and duplicate 100 μL aliquots were injected into the liquid chromatograph.

RESULTS AND DISCUSSION

The chemical structures of L,D-metyrosine and the TAGIT derivatizing reagent are shown in Fig 1. The retention times of the TAGIT derivatized L and D metyrosine methyl esters were 7.32 ± 0.04 and 8.73 ± 0.02 min, respectively (n=5). Retention factors (k') for the derivatized L and D

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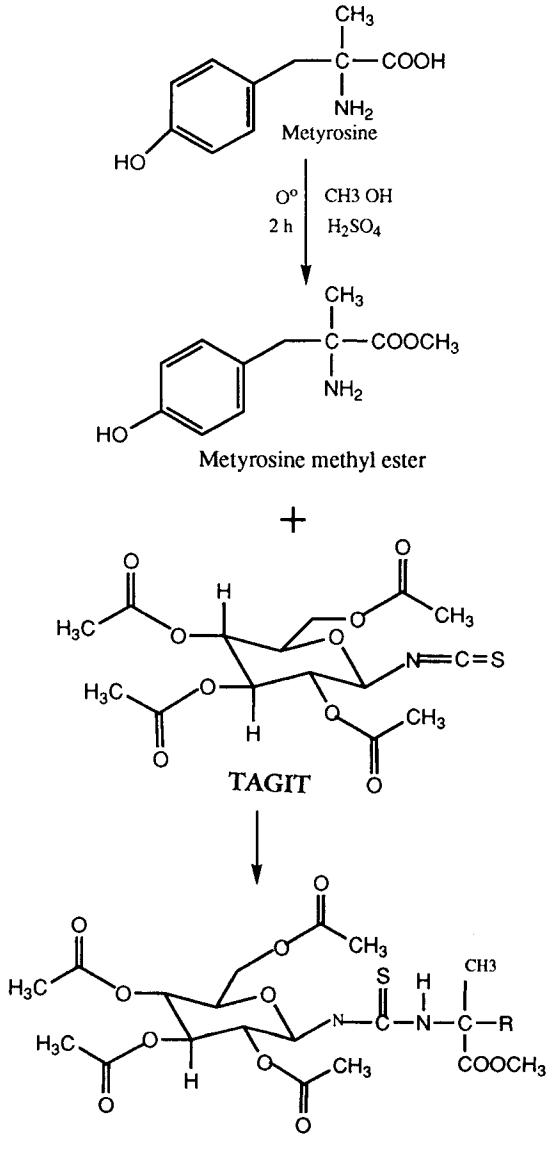

Figure 2. Proposed reaction of TAGIT with racemic metyrosine methyl esters to form diastereomeric thioureas.

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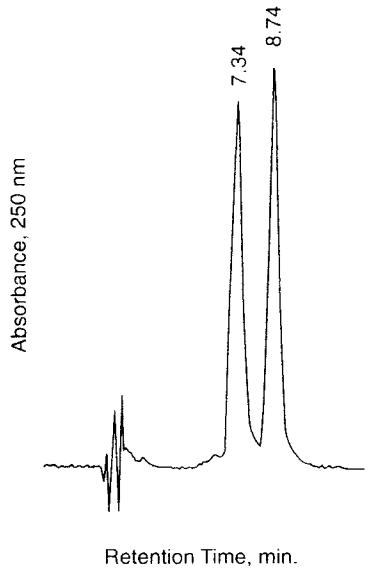

Figure 3. Separation of diastereomeric thiourea derivatives formed from racemic metyrosine methyl esters with TAGIT on an octadecylsilane column. Mobile Phase:Acetonitrile-Water (35/65 v/v) containing 0.1% triethylamine (pH 4.0 adjusted with trifluoroacetic acid); Flow rate 1 mL/min; detection at 250 nm.

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enantiomers were 2.3 ± 0.03 and 2.9 ± 0.04, respectively (n=5). The respective number of theoretical plates for the L and D enantiomers were 1508 ± 17 and 2106 ± 21 per 25 cm column [n=5] respectively. The resolution of the L and D-metyrosine derivatives, as expressed by the separation factor α, was calculated to be 1.26 ± 0.01 (n=5) . These data indicated that the method is suitable for separation of the derivatized metyrosine enantiomers.

The proposed mechanism for the reaction of TAGIT with racemic metyrosine methyl ester is shown in Fig. 2.[22] During the analytical development phase, the addition of TAGIT reagent greater than 50 μg/mL, resulted in a large negative peak which overlapped with the peaks of each enantiomer. It was found that adding hydrazine solution to the derivatization mixture aided in the removal of any excess TAGIT reagent. The effects of retention time and temperature on the TAGIT derivatization of the two enantiomers were evaluated. It was found that a 2 hr reaction time at 45°C resulted in maximum peak heights for the metyrosine derivatives. Fig. 3 shows a typical chromatogram of the separation of the diastereomeric thiourea

Table 1

Resolution of Diasteromeric Thiourea Derivatives Formed From Racemic Metyrosine* and TAGIT

Analyte	Mobile Phase Composition[a] (v/v) A	B	Rs	Retention Factor k_1	k_2
Metyrosine	65	35	1.99	2.30	2.90
	70	30	1.33	1.69	2.23
	75	25	1.03	1.37	1.87
	80	20	0.75	1.24	1.65
	90	10	0.62	0.95	1.20

* As the methyl esters.

[a] A = Water containing 0.1% Triethylamine pH 4.0 (adjusted with trifluoroacetic acid). B = Acetonitrile.

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derivatives of metyrosine methyl esters. The enantiomeric pairs were eluted in the sequence L, then D. Table 1 also shows the effect of organic modifier concentration in the mobile phase on resolution and retention of the metyrosine enantiomers.

Some investigators have found that the degree of separation of diastereomers on a normal phase column is largely dependent on the rigidity of their conformation.[17-19] Introduction of a bulky group as the ester moiety makes the conformation more rigid. For example, Nambara et al have reported that the neomenthylthiourea derivatives of amino acid tert-butyldimethylsilyl esters were completely resolved, whereas the corresponding methyl ester derivatives were not resolved.[18]

In summary, reversed phase chromatography coupled with methyl esterification and TAGIT derivatization facilitated baseline resolution of the metyrosine enantiomers. The conformations of the TAGIT diastereomers of metyrosine as the methyl esters are rigid owing to the bulky acetylglucosyl residue of TAGIT and bulkiness appears to favor baseline separation.

Good resolution may also be achieved with the derivatized metyrosine enantiomers due to the hydrophobicity of the derivatives which would interact favorably with the hydrophobic octadecylsilane stationary phase.

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