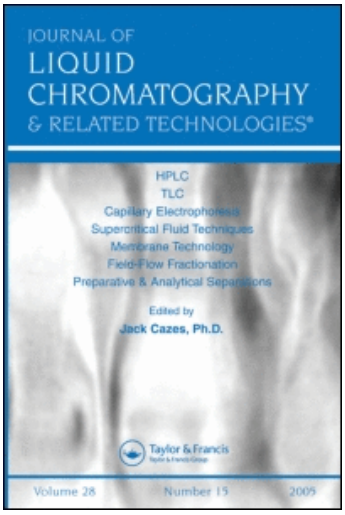

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LC Determination of the Enantiomeric Purity of L-Arginine Using a Teicoplanin Chiral Stationary Phase

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ABSTRACT

A direct enantioselective high-performance liquid chromatography (HPLC) method was employed successfully for determination of the enantiomeric purity of L-arginine. The elaborated method used teicoplanin macrocyclic antibiotic chiral stationary phase (CSP), known as Chirobiotic T, with a reversed-phase mobile phase consisting of methanol : 50 mM sodium dihydrogen phosphate buffer, pH 4.6 (2 : 8, v/v), at a flow rate of 1.0 mL/min and UV detection at 214 nm. Linearity, precision, accuracy, and the quantitation limit were determined. The method proved to be capable of

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determining 0.0025% (w/w) of D-arginine (the enantiomeric impurity) contrary to the pharmacopoeial limit measurement, in which only amounts of D-arginine higher than 1.5% (w/w) caused the measurement to fail.

Key Words: L-Arginine; Enantiomeric purity; Chiral HPLC; Chirobiotic T.

INTRODUCTION

Over the last two decades, chiral analysis has acquired increasing prominence, particularly in the pharmaceutical field, and chiral liquid chromatography has become a powerful and widely applicable analytical tool for this purpose.[1] High-performance liquid chromatography (HPLC) chiral separations can be achieved in several ways; using a chiral stationary phase (CSP) or chiral mobile phase additives (direct separation), performing derivatization with a chiral reagent to diastereoisomers followed by chromatography on an achiral column (indirect separation), or using chirooptical detection in combination with an achiral chromatographic system. Direct separations are preferred as they avoid derivatization with its possible difficulties.

Consequently, rapid progress in the development of CSPs has been made and a wide variety of CSPs are now available.[2] A new class of chiral selectors based on macrocyclic antibiotics has been introduced.[3] Their success can be attributed to the diversity of their structures that have multiple stereogenic centers and a variety of functional groups, which are known to provide multiple interactions necessary for enantioselectivity.[2,4–6] Indeed, they offer a unique combination of structural features useful in the interaction with chiral analytes. They are able to associate through (i) ionic interaction; (ii) hydrogen bonding; (iii) $\pi-\pi$; (iv) dipole–dipole interactions; and (v) hydrophobic interactions via the nonyl-tail chain.[7,8] The importance and superiority of macrocyclic antibiotics as chiral selectors, in comparison with other chiral selectors, is because they can be used in normal and reversed phases with greater stability and capacity.[2] The glycopeptide antibiotic CSP has great potential for the resolution of various chemical classes, including amino acids.[9–13]

Oral administration of L-arginine seems to cause significant subjective improvement in sexual function in men with organic erectile dysfunction by increasing the amounts of endogenous nitric oxide, where L-arginine is considered a substrate for nitric oxide synthase enzyme.[14,15] The penile erection requires the relaxation of the cavernous smooth muscle, which is triggered by nitric oxide.[16]

Amino acids are chiral compounds of great biological interest. Naturally occurring amino acids are L-enantiomers.[17] In the case of single enantiomer drugs, all other stereoisomers should be treated as any other organic impurities and the enantiomeric purity of such drugs should be controlled.[18]

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The pharmacopeias rely traditionally on potentiometric methods for control of optical purity of L-arginine.[19] However, potentiometric methods are known to be non-selective and often not sufficiently sensitive. To date, there has not been a published method dealing with the determination of traces of D-arginine in the presence of excess of L-arginine. In this work, an enantioselective HPLC method for determining the enantiomeric purity of L-arginine was elaborated and validated using a teicoplanin CSP column, commercially known as Chirobiotic T, as the chiral selector.

EXPERIMENTAL

Chemicals and Reagents

L-(+)- and D-(−)arginine, and the internal standard L-(+)-lysine were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Fairlawn, NJ). Analytical reagent grade sodium dihydrogen phosphate and phosphoric acid were obtained from BDH Chemicals (Poole, UK). L-Arginine capsules®, a dietary supplement (containing 500 mg of L-Arginine/capsule) was obtained from Natures Bounty Inc., Bohemia, NY, 11716.

Instrumentation and Chromatographic Conditions

The HPLC system consisted of a water solvent delivery pump (Model 510, Milford, MA), Waters injector with a 20 μL sample loop (Model WISP 710B), Lambda max model 481 LC spectrophotometry UV and a Hewlett–Packard 3394A integrator (Avondale, PA). The CSP used in this study was the macrolide-type antibiotic teicoplanin, known as Chirobiotic T (25 cm × 4.6 mm i.d.), which was purchased from Advanced Separation Technologies (Whippany, NJ). The mobile phase was methanol : sodium dihydrogen phosphate, pH 4.6 adjusted by phosphoric acid (2 : 8, v/v). The mobile phase was filtered through a Millipore membrane filter (0.2 μm) from Nihon Millipore (Yonezawa, Japan) and degassed before use. The flow rate was 1.0 mL/min and the chromatograms were monitored by UV detection at a wavelength of 214 nm.

Preparation of Standard Stock Solution

Stock solution of L-arginine, D-arginine, and internal standard L-lysine were prepared separately by accurately weighing 50 mg of each analyte, and

dissolving in methanol in 100 mL volumetric flasks; serial dilutions were carried out, using methanol, to obtain the concentration ranges of 0.5–20 μg/mL.

Preparation of Standard Solution of L-Arginine Capsules®

The contents of five capsules were weighed and powdered. An accurately weighed portion of the powder equivalent to 50 mg L-arginine was transferred to 100 mL volumetric flasks, methanol was added to the mark. The solutions were sonicated for 15 min centrifuged at 3000 rpm for 10 min and the supernatant was used to prepare solutions of various quantities of the analyte using methanol as dilutent.

Quantitation and Linearity

Equal volumes (20 μL) of the standard preparation and the assay preparations that contain L-arginine in methanol were injected into the chromatograph and the chromatograms were recorded. Calibration standards of each concentration were analyzed in triplicate. Calibration curves of L-arginine were constructed using ratio of the observed peak area of drug and internal standard vs. nominal concentrations of the drug. Least squares linear regression analysis of the data gave slope, intercept, and correlation coefficient data. From this data, a first order polynomial model was selected for the analyte.

Selectivity

The selectivity of the method was investigated by observing any interference encountered from excipients present in the formulations. It was shown that these components do not interfere with the proposed method.

Validation

The limit of detection (LOD) and the limit of quantitation (LOQ) were determined as three and ten times the baseline noise, respectively, following the United States Pharmacopoeia.[14] The results of the statistical analysis of the experimental data, such as the slopes, the intercepts, the correlation coefficients obtained by the linear squares treatment of the results, along with standard deviation of the slope (S_b) and intercept (S_a) on the ordinate, and the standard deviation of the residuals ($S_{y/x}$), was calculated and presented

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in the discussion. The good linearity of the calibration graphs and the negligible scatter of experimental points are clearly evident by the values of the correlation coefficient and standard deviation.

RESULTS AND DISCUSSION

Chromatography

The chemical structure of L- and D-arginine and L-lysine are shown in Fig. 1. In reversed phase HPLC, polar substances are weakly retained on the column and are eluted rapidly. Furthermore, aqueous buffers used as components of a mobile phase will permit direct introduction of aqueous samples into the column, as well as regulation of the extension by changing the mobile phase composition (pH, ionic strength, and content of organic modifier).[20] A successful resolution of the arginine enantiomers was achieved in the reversed phase mode on the teicoplanin glycopeptide antibiotics CSPs. Initial resolution of the enantiomers ($R_s = 1.5$) was obtained using a mobile phase consisting of 50 mM aqueous sodium dihydrogen phosphate : methanol, pH 5.0 (7 : 3, v/v). However, the achieved enantioresolution $R_s = 1.5$ was not sufficient for sensitive determination of the enantiomeric purity. Generally, resolution greater than 2 for the racemate is needed for quantitative trace analysis.[21]

The most significant parameters are pH of the mobile phase, concentration, and type of buffer.[3] The effect of pH was investigated with 50 mM sodium dihydrogen phosphate buffer in the range of 3–7, which is the stability range for macrocyclic glycopeptide antibiotics CSPs.[4] On the basis of these experiments, pH 4.6, which represents the optimal pH required for the enantioresolution, was chosen for further experiments. The effect of buffer concentration, which was studied in the range 20–100 mM sodium dihydrogen phosphate was pronounced. Through the use of higher buffer concentration (>70 mM), only a slight increase of retention time and enantioresolution were observed. At buffer concentration lower than 30 mM, poor peak shapes were observed. The buffer concentration of 50 mM was chosen as an optimum concentration. Changing the phosphate buffer with acetate buffer resulted in a high tailing factor. Two organic modifiers, methanol and acetonitrile, were examined in hydroorganic mobile phases that contained the 50 mM phosphate buffer (pH 4.6) as the aqueous component. Enantioseparation was achieved with both organic modifiers in the whole concentration range examined (10–40, v/v). As expected, the capacity factors decreased with increasing content of organic modifiers (more than 20, v/v), since the organic modifiers compete with the analytes for CSP resolution centers. These effects were more significant with acetonitrile, by replacing the acetonitrile modifier with

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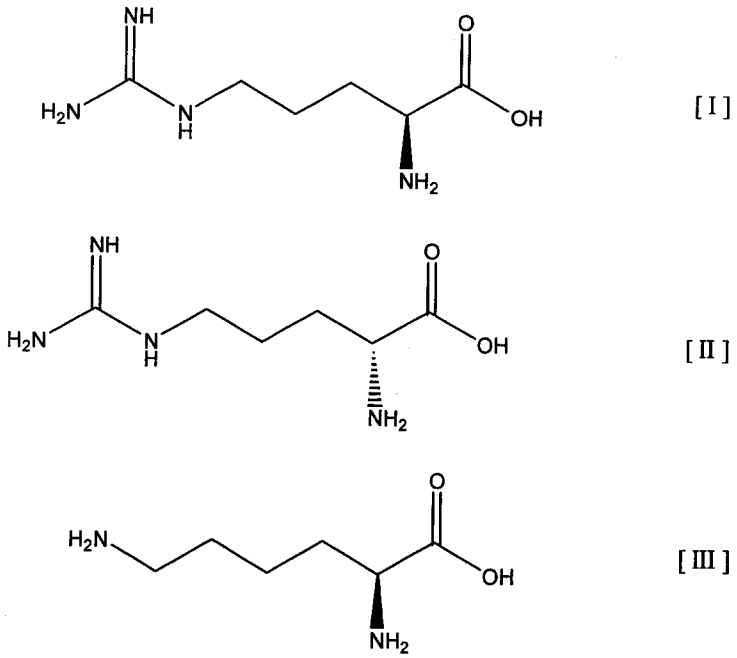

Figure 1. The chemical structures of L- and D-arginine [I and II] and L-lysine [III].

methanol; enhancement of the peak symmetry was observed, with improvement of the resolution of the enantiomers ($R_s > 3$) and reduced retention time (Table 1). The mobile phase containing methanol (20%, v/v) and 50 mM sodium dihydrogen phosphate buffer, pH 4.6, was chosen as the best mobile phase for the enantiomeric purity determination.

As Fig. 2(A) shows, sufficient enantioresolution ($R_s > 3.27$) in a short analysis time, was achieved under these conditions. The chromatogram shows the presence of 0.5% D-arginine in L-arginine after injection on a teicoplanin CSP. It is evident that very low concentrations of D-arginine can be detected and determined with reasonable precision.

Aboul-Enein and Ali[2] reviewed the possible bonding between the enantiomers and the macrocyclic glycopeptide antibiotics CSPs. The most important bondings involved are $\pi-\pi$ interactions, hydrogen bonding, inclusion complexation, dipole interactions, steric interactions, and ionic and cationic bondings. These bondings are a result of the complex structures of these CSPs, which contains sugar moieties and several aromatic rings, along with several chiral centers, inclusion baskets, hydrogen donors, and acceptor sites. It has been reported that these bonding sites are responsible for the surprising

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Table 1. Chromatographic parameter data for arginine enantiomers and internal standard using the proposed method.

			(Mean ± S.D., $n = 3$)	
Analyte	R_s	α[a]	k	t_r (min)
L-Lysine	Not calculated	1.51	1.14 ± 0.04	5.52 ± 0.11
L-Arginine	5.14	1.33	1.72 ± 0.06	7.12 ± 0.14
D-Arginine	3.27	Not calculated	2.28 ± 0.07	8.28 ± 0.07

[a]Separation factor (α), calculated as k_2/k_1.

chiral selectivities of these antibiotics. The studied enantiomers of arginine (Fig. 1) contain amino, imino, and carboxylic groups, and they have three p*K*a values (pK_1 2.18; pK_2 9.09; pK_3 13.2); at pH 4.6 of the mobile phase, the amino groups will be in the cationic form, which allows them to interact with complementary groups on the chiral selector (antibiotic). The inclusion baskets and the other functional moieties provide the chiral sites in which the enantiomers fit stereogenically in different fashion, which results in the chiral discrimination between the arginine enantiomers.

Linearity

In order to evaluate the suitability of the method for determining the enantiomeric purity of L-arginine, validation studies were carried out. The linearity of calibration curves (ratio of peak area of D/IS vs. concentration) for L-arginine in pure form, as well as dosage form, were checked over the concentration ranges of 0.5–20 μg/mL, with correlation coefficient (R^2) of better than 0.999 (Tables 2 and 3), as determined by least squares analysis.

Accuracy and Precision

The spiked samples of L-arginine that had compositions as shown in the second column of Tables 4 and 5, were prepared from L-arginine and D-arginine. The intra-day precision and accuracy as expressed by percent of relative standard deviation (RSD%) and percentage error were 1.11–2.45% and 1.02–2.06%, respectively, for pure form and 0.73–2.03% and 1.93–4.00% for dosage form, respectively. The inter-day precision and accuracy expressed by percentage RSD and percentage error were 0.97–2.35% and 0.69–1.92%,

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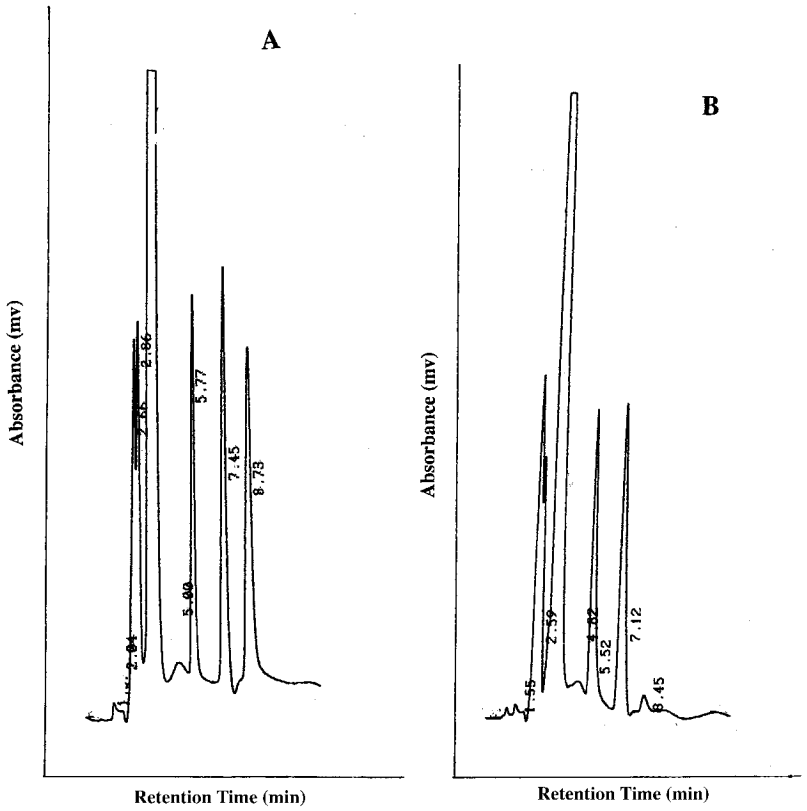

Figure 2. Chromatograms: (A) Separation of L-lysine (5.77 min), L-arginine (7.45 min), and D-arginine (8.73 min) in pure form. (B) Separation of L-lysine (5.52 min), L-arginine (7.12 min), and D-arginine (8.45 min) in capsules.

respectively, for pure form and 1.86–2.70% and 1.86–4.00% for dosage form, respectively. The detailed analytical data are shown in Tables 4 and 5.

LOQ and LOD

The LOQ and LOD were calculated with diluted solutions of L- and D-arginine. The LOQ was the level, which produced a relative standard deviation of about 10%. The LOQ was determined to be 0.5 μg/mL for both two enantiomers. The LOD was determined to be 0.25 μg/mL for L- and D-arginine, based on signal to noise ratio 3 : 1, respectively (Table 6).

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Table 2. Determination of L-arginine in pure form by the proposed method.

Analyte	Nominal concentration (μg/mL)	Measured concentration (μg/mL)	Recovery[a] (%)
L-arginine	0.5	0.51	102.0
	1.0	0.98	98.0
	3.0	3.05	101.6
	5.0	5.09	101.8
	10.0	10.05	100.5
	15.0	15.14	100.9
	20.0	19.75	98.9
Overall recovery			100.52
RSD (%)			1.42

[a]The results are the average of three separate determinations.

Application of the Analysis of D-Arginine in Pharmaceutical Formulation

The results of the validation study indicated that the method enabled reliable determination of the enantiomeric impurity D-arginine, as well as the active substance L-arginine. In order to verify applicability of the method for determining the enantiomeric purity of a dosage form, we performed

Table 3. Determination of L-arginine in pharmaceutical preparation by the proposed method.

Pharmaceutical preparation	Nominal concentration (μg/mL)	Measured concentration (μg/mL)	Recovery (%)
L-Arginine capsule[a]	0.5	0.51	102.0
(500 mg L-arginine/	1.5	1.52	101.3
capsule)	4.5	4.44	98.6
	7.5	7.45	99.3
	12.5	12.59	100.7
	17.5	17.62	100.7
			100.43
Overall recovery			1.15
RSD (%)			

[a]Product of Natures Bounty Inc. (NJ, USA).

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Table 4. Precision and accuracy of determination of D-arginine as an enantiomeric impurity in L-arginine standard.

Added amount of D-arginine (μg/mL)	Content of D-arginine (μg/mL)	Recovery (%)	RSD (%)	Error (%)
Intra-day[a]				
0.52	0.53 ± 0.013	101.9	2.45	1.92
1.45	1.48 ± 0.016	102.1	1.11	2.06
2.95	2.99 ± 0.035	101.4	1.17	1.02
Inter-day[b]				
0.52	0.51 ± 0.012	98.1	2.35	1.92
1.45	1.44 ± 0.016	99.3	1.11	0.69
2.95	2.98 ± 0.029	101.1	0.97	1.01

[a]Mean ± S.D. based on $n = 3$.
[b]Mean ± S.D. based on $n = 3$.

analogous validation experiments with L-arginine capsules. A typical chromatogram of a sample prepared from L-arginine capsule is shown in Fig. 2(B); the validation parameters obtained are presented in Table 5. The assessed recovery indicated that the accuracy of the method using the simple extraction procedure was excellent.

Table 5. Precision and accuracy of determination of D-arginine as an enantiomeric impurity in L-arginine capsule.

Added amount of D-arginine (μg/mL)	Content of D-arginine (μg/mL)	Recovery (%)	RSD (%)	Error (%)
Intra-day[a]				
0.25	0.26 ± 0.004	104.0	1.56	4.00
1.55	1.58 ± 0.032	101.9	2.03	1.93
3.75	3.84 ± 0.028	102.4	0.73	2.45
Inter-day[b]				
0.25	0.26 ± 0.006	102.0	2.35	4.00
1.55	1.59 ± 0.043	102.5	2.70	2.58
3.75	3.82 ± 0.071	101.5	1.86	1.86

[a]Mean ± S.D. based on $n = 3$.
[b]Mean ± S.D. based on $n = 3$.

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Table 6. Validation parameters for the determination of L-arginine using the proposed method.

Parameter	L-Arginine
Concentration range (μg/mL)	0.5–20
Intercept (a)	−0.0197
Slope (b)	0.9887
Correlation coefficient (R^2)	0.9997
$S_{y/x}$	0.0426
S_a	0.0224
S_b	0.0361
LOQ (μg/mL)	0.50
LOD (μg/mL)[a]	0.25

Note: $S_{y/x}$, standard deviation of residuals; S_a, standard deviation of the intercept; S_b, standard deviation of the slope.
[a]$S/N = 3$.

CONCLUSION

An enantioselective HPLC method that enables sensitive determination of the enantiomeric purity of L-arginine was developed. The method was found to be precise, accurate, sensitive, and well suited for routine control. The enantioresolution required for the sensitive determination of the enantiomeric purity, could be set easily by tuning the methanol content in the mobile phase; small changes in the buffer concentration and pH did not influence the separation significantly. The pharmacopoeial determination test for L-arginine proved to be, not unexpectedly, insensitive to a low level of D-arginine in the presence of a large excess of L-arginine. In conclusion, assessment of the enantiomeric purity of drugs requires the use of a state-of-the art analytical method. With the present broad range of available CSPs and advances in column technology, enantioselective HPLC can be considered as the method of choice.

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