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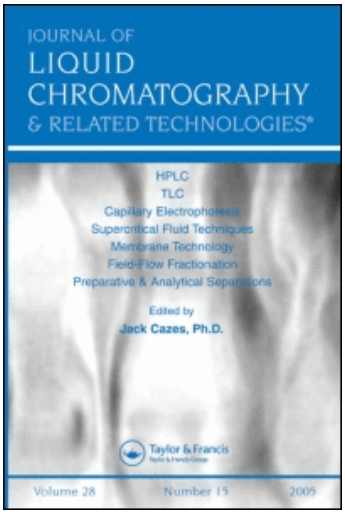

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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To cite this Article Rubio-Barroso, S. , Santos-Delgado, M. J. and Polo-Díez, L. M.(2008) 'Determination of D- and L-Amino Acids in Pharmaceutical Preparations by Indirect Chiral HPLC and Fluorimetric Detection', Journal of Liquid Chromatography & Related Technologies, 31: 18, 2851 — 2861
To link to this Article: DOI: 10.1080/10826070802388565
URL: http://dx.doi.org/10.1080/10826070802388565

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Journal of Liquid Chromatography & Related Technologies®, 31: 2851–2861, 2008
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ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070802388565

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Abstract: An indirect chiral HPLC method based on the derivatization reaction with o-phthaldialdehyde and N-acetyl-L-cysteine and fluorimetric detection is applied to determine the enantiomeric purity of amino acids in pharmaceutical preparations. Three samples, vanim™ 14, tebetane, and tulgrasum were analized and their enantiomeric contents were determined. Sample preparation did not require cleanup. The validity of the method was established. The D-AA content is not always specified in the labels of these pharmaceutical preparations.

Keywords: D, L-Amino acids, Derivatization reaction, Fluorimetric detection, HPLC, Pharmaceutical preparations

INTRODUCTION

As is known, molecular chirality is a fundamental phenomenom that plays an important role in biological processes. Enantiomeric purity of chiral compounds has become of great importance in pharmaceutical chemistry due to the potential toxicity of the non active enantiomeric form; in general, only one of the enantiomers presents the desired activity. In particular, the L-amino acids, L-AA, are of major importance in

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bioscience due to their beneficial effects; however, regarding the D-AA there are disagreements about their beneficial or harmful activity.[1] As it is known, the L-AA can racemise to their respective D-isomer by alkaline, heat treatment or microorganism action, which may take place during synthesis or the production processes.[1–3] Consequently, the development of analytical methods for the determination and control of the enantiomeric purity of AA continues to be of interest.[4–10]

Very different techniques have been proposed for enantiomer AA determination. Gas chromatography, GC, requires derivatization;[6,11,12] moreover, racemization at GC working temperatures may take place and the versatility of the methods is limited by the non contribution of the mobile phase.

Although techniques such as capillary electrophoresis, EC,[13] have also been used, they are considered, at present, less robust than high performance liquid chromatography, HPLC; the low sensitivity of spectrophotometric detection for most of the AA can be minimized by using the fluorimetric derivatization reagents.[1,4,5,8] HPLC chiral methods may be direct and indirect; direct methods[10,14–16] are based on the use of chiral bonded stationary phases, but no one may be considered as universal; so, indirect methods based on the use of C_{18} columns and chiral derivatization reagents to form the respective diastereoisomerics, may be an alternative when high purity derivatization reagents are available. Among the different reagents described in the literature, such as N- acetyl- L- cysteine, N- isobutyryl-D-cysteine, and R(-) and S(+)-4-(3-Isothiocyanatopyrrolidin-1-yl)-7-(NN-dimethylaminosulfonyl)-2,1,3-(benzoxadiazole), it seems that[1,4,5,8,17] N- acetyl- L- cysteine is preferred due to its easy handling and sensitivity.

In this paper, an indirect chiral HPLC method, previously described by us[1] has been optimized and used to determine the enantiomeric purity of the AA in pharmaceutical preparations. It is based on the derivatization reaction with o-phthaldialdehyde and N- acetyl- L- cysteine and fluorimetric detection. AA was analyzed by HPLC in several commercial products without cleanup; methodological details depend on the particular sample.

EXPERIMENTAL

Apparatus and Materials

A chromatographic system equipped with the following components was used: a Milton Roy CM 4000 high pressure gradient pump (Ribera Beach, FL, USA), a Rheodyne Model 7125 injection valve with a 20 μL

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loop (Cotati, CA, USA), a Perkin Elmer LS 30 luminiscence spectrometer (Norwalk, CT, USA), and a Milton Roy CI 4100 integrator. A Kromasil C-18 5 μm particle size column (150 × 4.6 mm id) (Análisis Vínicos, Ciudad Real, Spain), thermostated in a P-Selecta Presciterm bath (Barcelona, Spain) at 30°C was used for D, L-AA separation, and a Phenomenex safeguard column (Torrance, CA, USA) was also used. A P-Selecta ultrasonic bath and a P-Selecta Meditronic centrifuge able to apply 4800 rpm (3700 g) were used for the preparation of AA solutions and samples. Solvents for preparing the mobile phase were filtered through Phenomenex nylon membrane filters with 0.45 μm pore size and the sample extracts were also filtered using PTFE membrane filters, (13 mm, 0.5 μm pore size) (MFS, Dublin, CA, USA) inside a Visiprep vacuum manifold system (Supelco, Bellfonte, PA, USA).

Chemicals

AA used were: D- and L-serine (D and L-Ser), D and L-threonine (D and L-Thr), D and L-Alanine (D and L-Ala), D and L-Tyrosine (D and L-Tyr), D and L-Valine (D and L-Val), D and L-Tryptophan (D and L-Try), and D and L-Leucine (D and L-Leu) (Sigma, St. Louis, MO, USA and Fluka, Buchs, Switzerland). Standard aqueous solutions of D- and L-AA at concentration levels of 25 mgL^{-1} were prepared and stored in glass bottles at 4°C. O-phthalaldialdehyde (OPA), N-acetyl-L-cysteine (NALC) (Sigma and Fluka), and sodium borate (Scharlau, Barcelona, Spain) were used for the derivatization reaction. HPLC pure methanol (MeOH), tetrahydrofuran (THF), and sodium phosphate from Scharlau were used to prepare the mobile phase, which was degassed with helium (Carburos Metálicos, Barcelona, Spain). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

Samples

The following pharmaceutical preparations were analyzed: Vanim™ 14 solution (Fresenius Kabi; France S.A., Limognes, France), which is used for parental nutrition; Tebetane, capsules (Elfar-Drag, Madrid, Spain) used following gynaecological surgery and as prophylactic to prevent urinary complications; Tulgrasum, gauzes impregnated with oleous solution, 14 × 25 cm (Knoll S.A., Madrid, Spain) applied to burns, wounds, ulcers, dermatosis, etc. Their AA contents specified in the labels are shown in Table 3.

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Procedures

Sample Preparation

Vanim™ 14. A solution volume of 1 mL was diluted to 100 mL with purified water in a glass flask and filtered through a PTFE filter. The derivatization procedure was applied to 10 μL of this aqueous solution.

Tebetane. About 0.1 g of a capsule was dissolved in a few mL of purified water, diluted in a 100 mL glass flask, and shaken for 5 min in the ultrasonic bath. Then, the aqueous solution was filtered through a PTFE filter by means of the vacuum system. The derivatization procedure was applied to a volume of 20 μL of this solution.

Tulgrasum. The gauze was soaked in 100 mL of purified water for 24 hours and then shaken in the ultrasonic bath for 10 minutes to extract AA. The aqueous solution was filtered through a PTFE filter by means of the vacuum system. The derivatization procedure was applied to 20 μL of this solution. For recovery studies, D- and L-AA were added according to the expected preparation composition.

Derivatization Reaction. The derivatizing reagent was prepared by mixing 3 mg of NALC (dissolved in 2.5 mL of a 0.2 M sodium borate buffer solution, pH = 10.4) with 0.5 mL of the OPA aqueous solution, 6 mg L^{-1}. Then, 10–200 μL of the individual D, L-AA or their standard solution mixtures at 25 mg L^{-1} concentration level, or 10–20 μL of the prepared sample were added to the above buffer solution, diluting up to 2 mL. Afterwards, with the same buffer, 250 μL of the chiral derivatizing reagent were added and the mixture was agitated for 2 minutes by means of the ultrasonic bath.

HPLC Determination. The mobile phase was prepared by mixing a 12.5 mM sodium phosphate buffer solution, pH = 6.5 (solvent A), with MeOH/sovent A/THF (v/v/v): 100/60/6 (solvent B). The gradient profile applied is shown in Table 1, the flow rate being 1 mL min^{-1} and the temperature 30°C. The fluorimetric detection was carried out at 344 and 443 nm excitation and emission wavelengths, respectively. The injection volume of derivatized AA was 20 μL. The peak areas were used for quantification of AA. The retention times were used for identification purposes. In order to obtain the calibration of standard series, the above derivatization reaction was applied to all AA solutions or samples at concentration levels between 57–1240 μg L^{-1}.

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Table 1. HPLC method experimental conditions

	Variable	Optimized value
Derivatization reaction	OPA/NALC mol ratio	4/5
	OPA/AA mol ratio	100/1
	$V_{Methanol}$, mL	1
	Borato buffer, M	0.2
	pH	10.4
	$t_{reaction}$, min	2
Chromatographic separation	Column	Chromasil C-18 (150 × 4.6 mm), 5 μm
	Mobile phase*	Solution A: phosphate buffer 12.5 mM (pH, 6.5) Solution B: Methanol/ Solution A/THF 100/60/6, (v/v/v/)
	T, °C	30
	q, mL min^{-1}	1
	λ_{exc}, λ_{em}	344, 443

	Time, min	%A ($H_2PO_4^-$/ HPO_4^{2-})	% B (MeOH/$H_2PO_4^-$/ HPO_4^{2-}/THF)
*Mobile phase gradient	0	78	22
	2	78	22
	5	60	40
	7	45	55
	15	40	60
	23	0	100
	25	78	22

OPA, o-phthaldialdehyde; NALC, N-acetyl L-cysteine; THF, tetrahydrofuran; $t_{reaction}$, reaction time (min); q, flow rate (mL min^1).

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RESULTS AND DISCUSSION

HPLC Method

According to the composition specified in the labels of the pharmaceutical preparations, a study by conventional HPLC was first made on standard solutions containing the expected AA and applying the proposed method. The optimum experimental conditions are summarized in Table 1. The elution order was the following: D, L-serine, D,L-threonine,

D,L-alanine, L,D-tyrosine, L,D-valine, L,D-tryptophan, and D,L-leucine. Figure 1 shows a representative chromatogram obtained in these conditions. Afterwards, a screening on the pharmaceutical samples was made; the AA were identified by their retention times and the area increased after spiking the samples.

Analytical Characteristics of the Method for Standards

Table 2 shows the analytical characteristic of the method for standards. The calibration graphs of the standards series in the concentration range

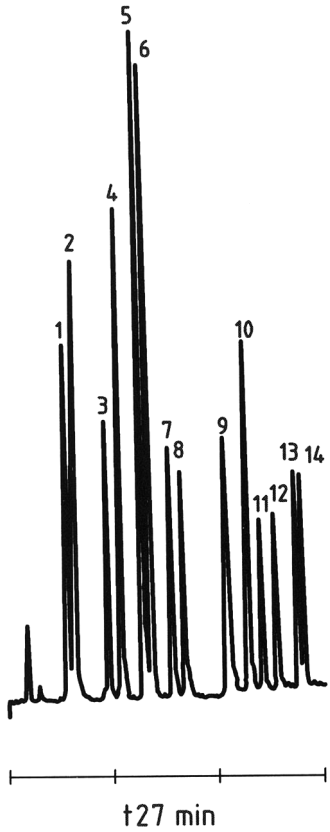

Figure 1. Chromatogram of a seven D,L-AA standard mixture. Kromasil C-18 column (150×4.6 mm, 5 μm); gradient of the mobile phase, see Table 1; flow rate 1 mL $min^{-1;}$ temperature 30°C; injection volume 20 μL at concentration levels between 342–435 $\mu gL^{-1;}$ $\lambda_{ex}/\lambda_{em}$: 344/433 nm. Peaks: 1, D-Ser; 2, L-Ser; 3, D-Thr; 4, L-Thr; 5, D-Ala; 6, L-Ala; 7, L-Tyr; 8-D-Tyr; 9, L-Val; 10, D-Val; 11, L-Trp; 12, D-Trp; 13, D-Leu; 14, L-Leu.

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Table 2. Analytical characteristics of the method for standards

AA	c, $\mu g\ L^{-1}$	RSD (%)	DL, $\times 10^{-3}\,\mu g\,mL^{-1}$	QL, $\times 10^{-2}\,\mu g\,mL^{-1}$	t_R, min	RSD (%)
D-Serine	128	3.8	6.0	2.0	5.12	3.8
L-Serine	128	2.3	1.5	0.4	5.78	3.6
D-Threonine	128	3.2	7.5	2.5	8.52	2.1
L-Threonine	128	2.5	1.0	0.3	9.73	1.4
D-Alanine	133	3.8	4.0	0.1	11.7	1.1
L-Alanine	133	2.4	1.0	0.3	12.3	1.1
L-Tyrosine	133	4.3	5.5	1.7	14.0	1.0
D-Tyrosine	133	5.5	5.0	1.6	15.0	1.2
L-Valine	142	2.9	9.0	3.0	18.0	1.4
D-Valine	142	3.6	2.0	0.7	20.0	1.2
L-Triptophan	155	3.3	8.5	2.8	21.5	0.85
D-Triptophan	155	3.0	4.5	1.4	22.9	0.68
D-Leucine	111	3.2	6.0	2.0	24.4	0.51
L-Leucine	111	3.8	5.0	1.7	24.9	0.50

RSD, relative standard deviation (n = 4) ; DL, detection limit. QL, quantification limit.

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between 111–430 $\mu g\,L^{-1}$ showed linear regression coefficients between 0.997–0.999; their slopes were quite similar to those of the standard adition method, which were also linear in the concentration range studied; so matrix effects were not significant. The RSD values at the concentration level in the range 111–142 $\mu g\,L^{-1}$ were lower than 6% (n = 4). Detection limits (DL), defined as three times the signal to noise ratio (3S/N) and quantification limits QL = (10 S/N) were lower than 0.009 and 0.030 $\mu g\,mL^{-1}$, respectively; retention times and their RSD (n = 4) are also shown.

Determination of D,L-AA in Pharmaceutical Preparations

The method for determination of D, L- AA in pharmaceutical samples described in the experimental section was applied. Table 3 shows the results obtained. The chromatograms for vanim, tebetane, and tulgrasum samples are shown in Figure 2.

Several AA were detected in vanim as their L-form but none in D-form; so, in the vanim preparation L-AA were quantified; the precision given by the relative standard deviation (RSD, %) is also indicated. In general, the RSD % values were lower than 10% (n = 4).

In the case of tebetane, it contains only L-Ala, which is in agreement with specifications. Precision was of 2% (n = 4).

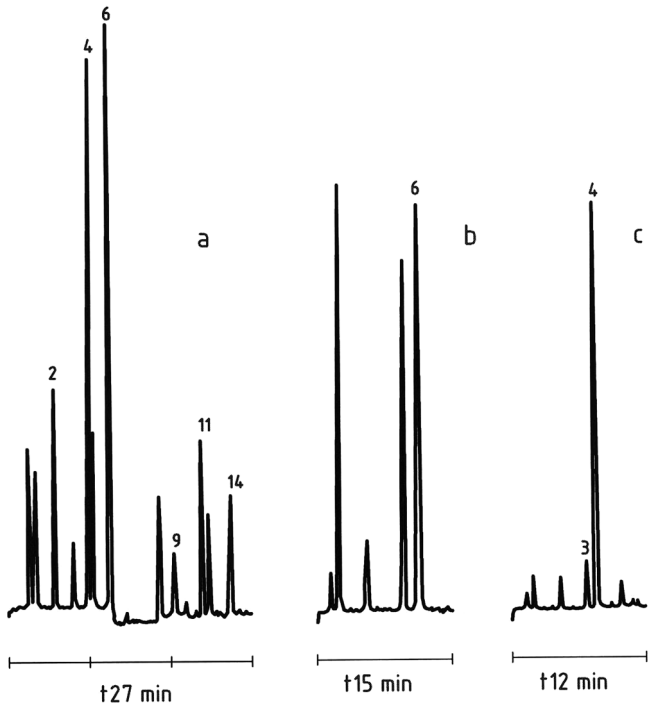

Figure 2. Chromatograms of the samples: a) Vanim™ 14, b) Tebetane c) Tulgrasum. Kromasil C-18 column (150 × 4.6 mm, 5 μm); gradient of the mobile phase, see Table 1; flow rate 1 mL min^{-1}; temperature 30°C; injection volume 20 μL, $\lambda_{ex}/\lambda_{em}$: 344/433 nm. Peaks: 2, L-Ser; 3, D-Thr; 4, L-Thr; 6, L-Ala; 7, L-Tyr; 9, L-Val; 11, L-Trp; 14, L-Leu.

Regarding tulgrasum, the enantiomer ratio was not specified in the preparation. However, as Table 3 shows, both forms were detected and determined, being the enantiomeric ratio (ER = D/L) of 0.22. The total D, L-Thr amount was quite close to that specified in the preparation. Although disagreements about the toxicity of D-AA exist,[1] according to the literature D-threonine seems to have a harmful activity, affecting the neurotransmitter balance in the brain, which may have consequences during early postnatal life.[18]

To determine the validity of the method, a recovery study was carried out (see Table 4).

The vanim sample was spiked with a mixture of the L-AA detected at three concentration levels, in the range 0.240–0.680 mgL^{-1}; recoveries were between 88–102% for L-Trp and L-Leu, respectively, with RSD values lower than 8% (n = 4).

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Table 3. Determination of D,L–AA in pharmaceutical preparations

Pharmaceutical preparation	Peak n°	AA	C, Specified	C, Found	RSD* (%)	Error (%)
Vanim® 14 (solution)	2	L-Ser	0.34**	0.37**	10	8.8
	4	L-Thr	0.42	0.45	12	7.1
	6	L-Ala	1.2	1.1	5	8.3
	7	L-Tyr	0.017	0.02	9	18
	9	L-Val	0.55	0.52	4	5.4
	11	L-Trp	0.14	0.12	2	14
	14	L-Leu	0.59	0.62	0.3	5.1
Tebetane (capsules)	6	L-Ala	100***	95.7***	2	4.3
Tulgrasum (gauzes)	3	D-Thr		2.6****	6	
	4	L-Thr		11.8	4	
		D, L-Thr	15.8****	14.4		8.9

*n = 4; **c = g/100 mL; ***c = mg/capsule; (capsule amount = 0.5527 g); ****c = $\mu g/cm^2$; gauze (14 × 25 cm).

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Recovery for tebetane was around 95% at the concentration level of 1 mg g^{-1}, with RSD of 5% (n = 4).

In the case of tulgrasum, the recoveries were 94 and 97% for D- and L-Thr, respectively, with RSD values lower than 8% (n = 4).

Table 4. Recovery study of D,L-AA in pharmaceutical preparations

	AA	c* (mg L^{-1})	RSD (%)	R (%)	c* (mg L^{-1})	RSD (%)	R (%)	c* (mg L^{-1})	RSD (%)	R (%)
Vanim 14®	L-Ser	0.30	5	96	0.480	6	95	0.666	0.6	102
	L-Thr	0.30	2	93	0.480	3	94	0.666	1	96
	L-Ala	0.25	1	92	0.410	0.5	101	0.562	0.5	101
	L-Tyr	0.31	1	94	0.498	0.7	93	0.684	1	95
	L-Val	0.28	6	89	0.444	8	92	0.610	5	94
	L-Trp	0.24	8	91	0.391	2	88	0.537	0.8	93
	L-Leu	0.24	7	92	0.391	3	102	0.537	2	99
Tebetane	L-Ala	1**	5	95						
Tulgrasum	D-Thr	10***	6	94						
	L-Thr	10***	7	97						

*concentration of added AA mixture, **mg g^{-1}, ***$\mu g\,cm^{-2}$.

CONCLUSIONS

The validity of an HPLC indirect chiral method for the determination of AA enantiomeric ratios in pharmaceutical preparations has been shown. Sample preparation is easy, although, it varies slightly depending on the nature of the sample. The D-enantiomer was detected in one of three preparations analyzed. Low amounts of the D-isomer can be determined in the presence of large L-enantiomer amounts. The importance of controlling enantiomeric purity of AA in pharmaceutical preparations is clear because this information is not always specified in their labels.

ACKNOWLEDGMENT

The financial support of the Spanish DGICYT project PQU2003–00667 is gratefully acknowledged.

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Received January 19, 2008
Accepted February 22, 2008
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