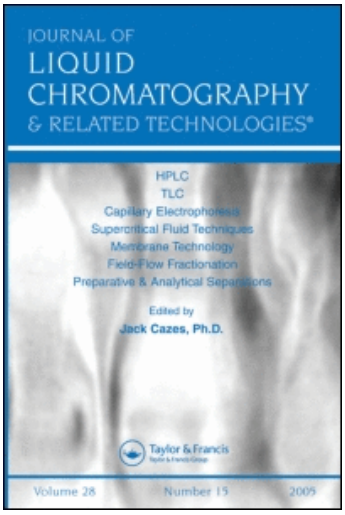

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DETERMINATION OF RACEMIC THALIDOMIDE IN HUMAN PLASMA BY USE OF AN AVIDIN COLUMN AND SOLID PHASE EXTRACTION

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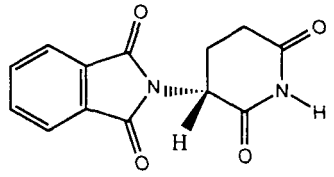
ABSTRACT

A HPLC method was developed to determine racemic thalidomide in plasma on a avidin protein column using solid phase extraction. The enantiomers were separated isocratically using a mobile phase of 2:98 v/v 2-propanol: phosphate buffer (0.1M, pH 4) at a flow rate of 0.6 mL/min at ambient temperature with detection at 300 nm. The chromatographic run time was less than 13 min. Calibration curves were prepared for each enantiomer in the range 100-5000ng/mL. The correlation coefficients for each enantiomer were greater than 0.999. The method is sensitive and can be used to measure plasma blood levels (100-1000ng/mL) of each enantiomer. The % RSD for quality control samples for both enantiomers were less than 10%. A C-18 SPE cartridge was used to extract the drug from plasma and recoveries of the enantiomers were greater than 95%. The limits of quantitation and detection were 100ng/mL and 50 ng/mL (s/n >3), respectively, for each enantiomer. Two of the commercially available metabolites of thalidomide were found not to interfere with the assay procedure.

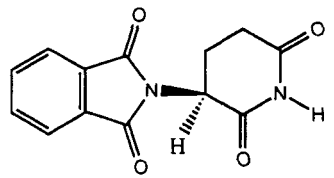
INTRODUCTION

Thalidomide (alpha-phthalimidoglutarimide) is an excellent therapeutic agent with almost no toxicity. It was introduced in 1956 as a sleep inducing agent, but was later withdrawn in 1961 because of its toxic effect on fetus. The recent renewed interest of various companies in thalidomide is based on its potential to treat inflammatory disorder associated with leprosy[1] and graft vs host disease(GVHD)[2] and to treat mouth, throat and rectal ulcers[3] associated with the AIDS patient. Thalidomide exists as a racemic mixture of R(+) and S(-) enantiomers. There are contradictions in the literature over the toxicological differences of the enantiomers. Blaschke et al.[4] has reported that S thalidomide is teratogenic in rats and mice, but Fabro et al.[5] found that both enantiomers were teratogenic in rabbits. The mechanism of fetal toxicity of thalidomide is still unknown. To investigate toxicity of thalidomide, an analytical method is required which can quantitate both enantiomers in plasma/blood. The paucity of pharmacokinetic data for the individual enantiomers is mainly due to the lack of sensitive stereo-selective analytical methods for thalidomide[6]. In September 1997, the Dermatologic and Dental Drugs Advisory Committee of FDA recommended the approval of thalidomide for *erythema nodosum leprosum,* a serious inflammatory condition in leprosy patients. Thalidomide has never been previously approved for use in the United States.

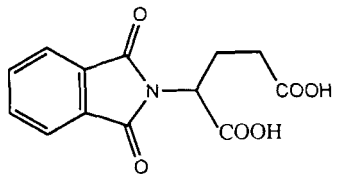
A number of analytical methods have been reported for quantitating racemic thalidomide from plasma using high performance liquid chromatography and gas chromatography[7]. Most methods reported are not stereo-selective. Eriksson et al.[8] have determined racemic thalidomide in blood and plasma by HPLC without degradation. Chen et al[9] have reported a HPLC method to determine racemic thalidomide in urine and plasma. Delon et al.[10] have developed a rapid and sensitive HPLC method using UV detection to determine thalidomide in human plasma. Reepmeyer[11] separated R(+) and S(-) thalidomide by HPLC using reversed phase chromatography and Aboul Enein et al.[12] have separated racemic thalidomide by HPLC using a cellulose tris-4-methyl benzoate chiral stationary phase. At the present, only two stereo-selective methods are available for the quantitation of thalidomide enantiomers in plasma[13,6]. One of the methods[13] did not define the dynamic range of quantitation and the other method[6], although sensitive uses liquid-liquid extraction and has a chromatographic run time of 20 min. The specificity of the latter method against interferences by the metabolites was not mentioned. The method herein can measure blood levels (100-5000 ng/mL) of each enantiomer of thalidomide with an analysis run time of less than 13 min. In addition, the method uses a solid phase extraction clean up which is less laborious and environmentally more friendly than liquid-liquid extraction. Two of the commercially available metabolites of thalidomide were checked for interferences with the parent drug. Figure 1 shows the chemical structures of thalidomide and its two available metabolites.



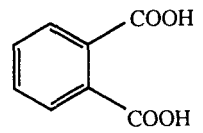
R-(+)-Thalidomide



S-(-)Thalidomide



N-Phthaloyl-L-glutamic acid



Phthalic acid

Figure 1. Chemical structures of thalidomide and two metabolites

Direct separation of enantiomers using chiral columns or chiral additives in the mobile phase is becoming increasingly popular for the analysis of chiral pharmaceuticals. In this paper, the separation of thalidomide enantiomers was achieved on a newly introduced, commercially available protein column (Biotic AV-1). The packing contains an avidin protein covalently bound to a hydrophobically-derivatized silica surface and is an internal surface reversed phase column particularly suited for chiral separations[14]. The stability of the column over the past six months has been very satisfactory.

EXPERIMENTAL

Reagents and Chemicals

Racemic thalidomide, the R and S enantiomers and N-phthaloyl L-glutamic acid were obtained from Research Biochemical Incorporated (Natick, MA). Phthalic acid and labetalol were purchased from Sigma (St Louis, MO). Diethyl ether and 2-propanol were purchased from. J. T. Baker (Phillipsburg, NJ) and Fisher Scientific (Fair Lawn, NJ). Freshly distilled water was used in mobile phase preparation. Drug free human plasma was obtained from

Biological Specialty Corp (Colmar, PA). Bioptic AV-1 column and its guard column were obtained from Meta Chem Technologies (Torrance, CA). The octadecylsilane SPE (1cc/100mg) cartridges were obtained from Varian Sample Preparation Products (Harbor City, CA).

Instrumentation and Chromatographic Conditions

The HPLC system consists of a Beckman pump (Model 110A), a Rheodyne 7125 injector equipped with a 50 μL loop and a UV/Vis detector (Waters,Model 481). The chromatogram was recorded on a Shimadzu integrator (Model C-R3A, Chromatopac). The separation was achieved on a Bioptic column (150 mm x 4.5 mm I.D) with a mobile phase of 2-propanol-phosphate buffer (pH4, 0.1M)-2:98 v/v at a flow rate of 0.6 mL/min and at ambient temperature (22±1°C). The Bioptic AV-1 column was protected with a guard column containing the same stationary phase and a frit filter coupled on line. The detection wavelength was set at 300 nm. A Vac Elut manifold purchased from Analytical International was used for the solid phase extraction.

Preparation of Standard and Spiked Solutions

Stock solutions of 100 μg/mL and 10 μg/mL of each enantiomer of thalidomide were prepared in methanol and stored at -20°C. Fresh stock solutions were prepared monthly. A stock solution of 100 μg/mL of labetalol (Internal standard) was prepared in methanol and stored at 4°C. One mL of plasma and 1 mL of phosphate buffer (0.025M, pH 2.5) were added to a test tube and the mixture vortexed and spiked with aliquots of both enantiomers to provide concentrations in the 100-5000 ng/mL range. The internal standard (labetalol) was added to give a concentration of 5 μg/mL of labetalol in plasma. The spiked samples were vortexed and kept on ice until further analysis by solid phase extraction.

Solid Phase Extraction

A C-18 SPE cartridge was conditioned by one column volume each of methanol and water. Then the spiked plasma was applied onto the cartridge with a vacuum less than 20 KPa. The cartridge was washed with two column volumes of water and dried under vacuum at more than 80 KPa. The analytes were eluted with 1 mL of anhydrous diethyl ether without application of any vacuum. The eluent was evaporated with the aid of a nitrogen stream and the residue was reconstituted in 130 μL of mobile phase. Fifty μL of the reconstituted solution was injected into the liquid chromatograph.

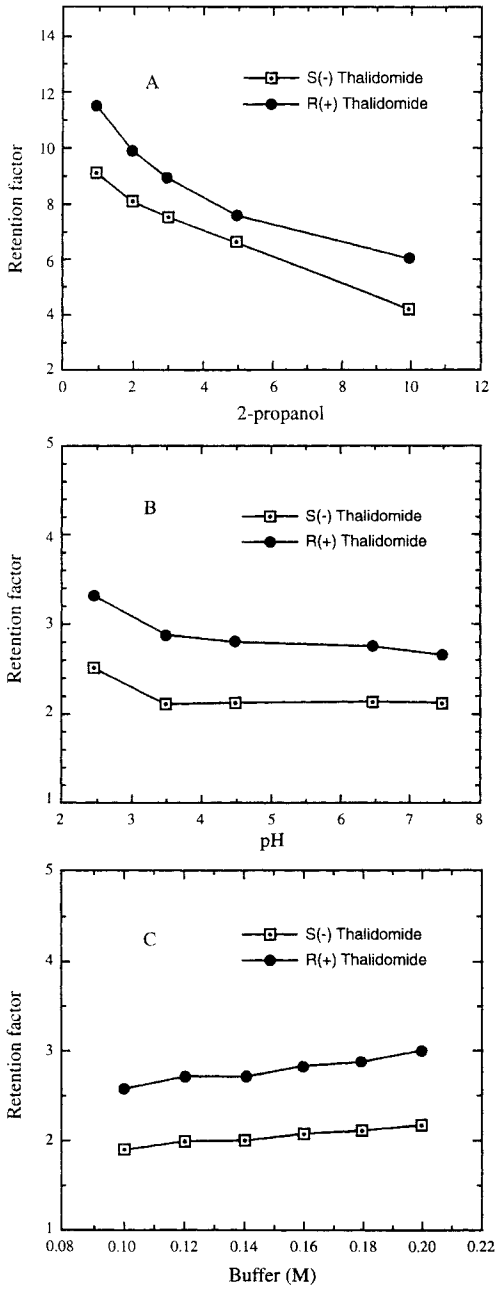
RESULTS AND DISCUSSION

The retention of a drug on a protein column is strongly influenced by the organic modifier, pH, buffer strength, and anions in the mobile phase. The assay method for thalidomide designed herein was developed on a column in which avidin protein is chemically bonded to silica gel by a hydrophobic group. Avidin, a basic protein with an isoelectric point between 9.5 - 10, has functional groups such as aspartic acid, glutamic acid and histidine in its structure. The ionization states of these functional groups depend on the pH of the mobile phase. The retention time of an acidic drug with carboxyl functions increases on the avidin column as the pH of the mobile phase is increased because an ionized acidic drug undergoes electrostatic interaction with the positively charged protein phase.

The opposite is true with an amine type drug. The retention and enantioselectivity on the protein column is thought to be due to both electrostatic and hydrophobic interactions between drug and stationary phase. Other nonspecific interactions such as hydrogen bonding and charge transfer interaction also play a vital role in the binding and chiral recognition of a drug molecule by the protein column.

The effects of organic modifier, pH and buffer strength on the retention of thalidomide enantiomers on the avidin column were investigated. Methanol, 2-propanol and acetonitrile at concentrations of 2% in the mobile phase effected baseline separation of the enantiomers with resolution values of 2.32, 2.20 and 2.53, respectively. Methanol showed the longest retention time compared to either 2-propanol or acetonitrile. However, considering retention time and peak shape, 2-propanol was selected as the organic modifier for the separation of the thalidomide enantiomers. At 5% 2-propanol in the mobile phase, resolution between the two enantiomers was lost. The effect of 2-propanol (1-10%) on the retention of thalidomide enantiomers is shown in Fig. 2A.

The pH of the mobile phase was varied in the 2.5-7.5 range (Fig. 2B). The retention of thalidomide enantiomers was not significantly influenced by the pH of the mobile phase. This was expected because thalidomide remains mostly unionized in the pH range studied. The retention times of the enantiomers increased slightly below pH 3. This may be due to a change in protein conformation below pH 3. Also, the phosphate buffer strength of the mobile phase was varied in the 0.1-0.2 M range (Fig. 2C). It was found that there was no significant influence of buffer strength on the retention of thalidomide enantiomers. Thus, 2-propanol:phosphate buffer (pH 4, 0.1M) 2:98 v/v was selected as the mobile phase to separate the thalidomide enantiomers with retention times for S(-) and R(+) thalidomide of 8.4 and 10.5 min, respectively (Fig. 3).



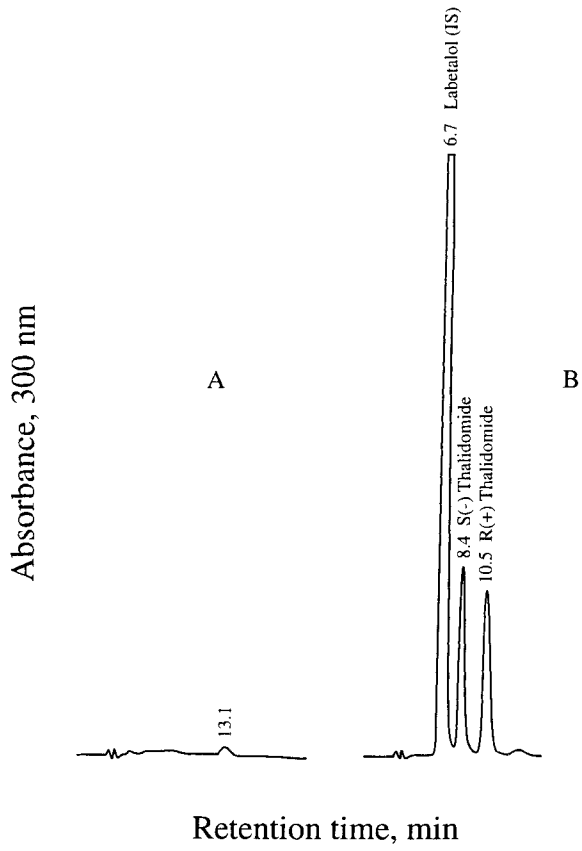


Figure 3. Chromatograms of A) blank serum and B) spiked plasma containing 600 ng/mL each of S(-) thalidomide and R(+) thalidomide and 5 μg/mL of labetalol on the avidin column. Mobile phase: 2-propanol- buffer (0.1M, pH4) 2:98 v/v, flow rate of 0.6 mL/min, detection at 300 nm and ambient temperature.

Figure 2 (left). Effect of mobile phase compositions on the capacity factor of thalidomide on avidin column A] Effect of 2-propanol on the retention factor of S(-) thalidomide and R(+) thalidomide on avidin column; Mobile phase was 2:98 v/v 2-propanol-aqueous phosphate buffer (pH 4, 0.1M). B] Effect of mobile phase pH on retention factor of S(-) thalidomide and R (+) thalidomide on avidin column; Mobile phase was 2:98 v/v 2-propanol-aqueous phosphate buffer (0.1M). C] Effect of buffer concentration in the mobile phase on retention factor of S(-) thalidomide and R(+) thalidomide on avidin column; Mobile phase was 2:98 v/v 2-propanol-aqueous phosphate buffer (pH 4), flow rate of 0.6 mL/min, detection at 300 nm and ambient temperature.

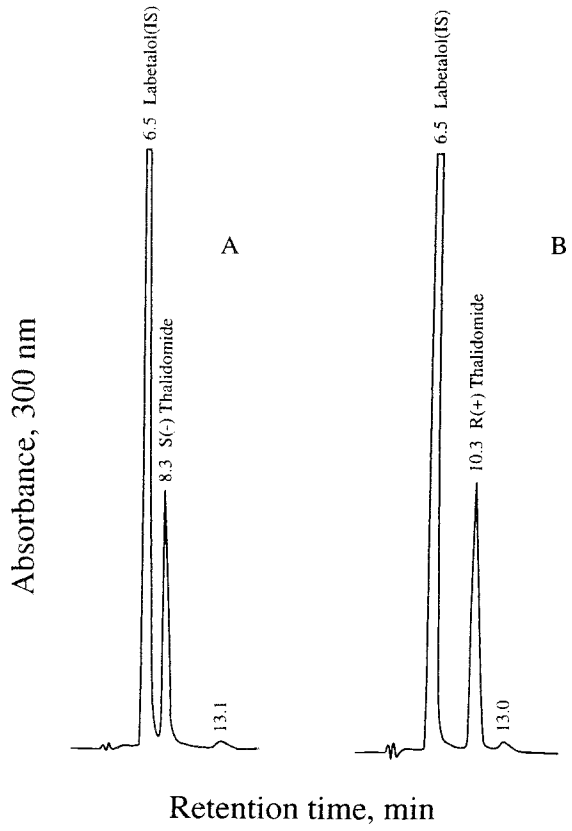


Figure 4. Extraction of A] S (-) thalidomide and B) R (+) thalidomide from human plasma by solid phase extraction and high performance liquid chromatography. Mobile phase: 2-propanol: buffer (0.1M, pH4) 2:98 v/v, flow rate of 0.6 mL/min, detection at 300 nm and ambient temperature.

Since, the retention and resolution of thalidomide enantiomers were not affected by either pH or buffer strength of the mobile phase, it is likely that both retention and enantioselectivity of thalidomide on the avidin column depends on hydrophobic and other nonspecific interactions with the stationary phase.

It has been reported that thalidomide undergoes degradation in aqueous solution.[8,10] It was reported by various authors that the rate of degradation of the drug was reduced significantly by acidifying the solution and reducing the temperature. The drug was found to be stable in plasma for at least six hours at 4°C.[10] To extract racemic thalidomide from plasma, C_{18}, C_8, C_1 and silica SPE

cartridges were investigated. Thalidomide was retained by all the cartridges investigated, but almost quantitative recovery was only obtained with C_{18} cartridge. Thalidomide is also known to undergo racemization at physiological pH.[8]

Although studies[13] have been performed on racemization of thalidomide in pH 7.4 phosphate buffer and citrated human and rabbit plasma, detailed studies on the influence of various pH and buffers on racemization of thalidomide were not reported. Eriksson et al.[6] have used Sorensen's buffer (0.025 M,pH 1.5) to stabilize blood samples containing thalidomide before extraction with diethyl ether.

In our extraction procedure, we used phosphate buffer (0.025M, pH2.5) and cooled the plasma samples in ice to stabilize plasma samples prior to extraction. The spiked plasma were processed as quickly as possible. To check if there was any racemization during the extraction process, each individual enantiomer was extracted by solid phase extraction and injected into the HPLC system. The chromatograms in Figure 4 show that no racemization occurred during extraction of the individual enantiomers from plasma by solid phase extraction.

Calibration curves were prepared for both enantiomers in the 100-5000 ng/mL range in plasma. Typical correlation coefficients, slopes and intercepts of the calibration curves are listed in Table 1. The % RSD of the spiked samples for both enantiomers were less than 10% (Table 2). The limits of quantitation and detection for the enantiomers were 100 ng/mL and 50 ng/mL (s/n >3), respectively. The intraday and interday accuracy and precision for both enantiomers were less than 11% and 15%, respectively, in the concentration range studied.

The recoveries of the enantiomers by solid phase extraction were determined by comparing peak height of extracted analyte from spiked plasma samples to those obtained from the unextracted standard solution (pH 2.5). The recoveries were 110± 2.1 and 105± 2.6 % at 0.5 μg/mL and 1 μg/mL, respectively, for S(-) thalidomide and 107 ± 3.1 and 98± 2.7 % at 0.5 μg/mL and 1 μg/mL, respectively, for R(+) thalidomide.

While there are four reported metabolites of thalidomide,[15] only two of the metabolites, N-phthaloyl L-glutamic acid and phthalic acid, are commercially available. They were injected into the HPLC and checked for interferences with the thalidomide enantiomer peaks. Both metabolites showed longer retention times than either thalidomide enantiomers. The retention times for N-phthaloyl L-glutamic acid and phthalic acid were 19 and 21 min, respectively.

Table 1

Typical Linear Regression Data for S(-) -Thalidomide and R(+)-Thalidomide

Analyte	Concentration Range (ng/mL)	r (n=6)	Intercept	Slope
S(-)Thalidomide	100-5000	0.9991	0.0303	0.0608
R(+)Thalidomide	100-5000	0.9992	0.0270	0.0544

Table 2

Percent Error and RSD of Spiked Plasma Samples of S(-)-Thalidomide and R(+)-Thalidomide

Analyte	Concentration Added, μg/mL	Concentration Found μg/mL*	Error (%)	% RSD
S(-)Thalidomide	0.70	0.77±0.03	10.0	4.0
	1.50	1.54±0.04	2.7	2.6
	4.00	4.32±0.05	8.0	1.15
R(+)Thalidomide	0.70	0.76±0.02	8.57	2.63
	1.50	1.55±0.06	3.33	3.87
	4.00	4.37±0.05	9.25	1.14

* Mean and std. dev.; based on n=3.

Figure 5 shows the chromatogram of thalidomide and N-phthaloyl L-glutamic acid. Phthalic acid is detected only at high concentration (100 μg/mL). The metabolites have longer retention times than thalidomide presumably due to the stronger interaction with the avidin protein caused by the presence of free carboxylic acid groups in their structures (Fig 1). Since the other two unavailable thalidomide metabolites are structurally similar to the N-phthaloyl L-glutamic acid metabolite, it is unlikely that these two metabolites will interfere with the assay procedure.

The stationary phase contains an avidin protein covalently bonded to a hydrophobically derivatized silica surface. The binding capacity of the protein phase can be altered by changing the conditions of pH and organic modifier

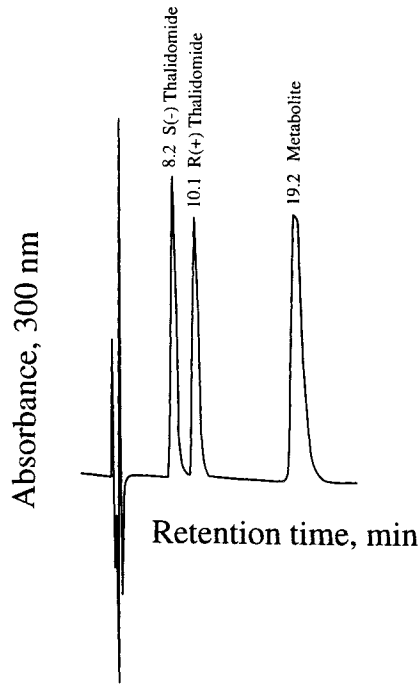


Figure 5. Chromatogram of racemic thalidomide with one of the known metabolites, N-phthaloyl L-glutamic acid, on the avidin column with a mobile phase of 2-propanol-buffer (0.1M, pH4) 2:98 v/v, flow rate of 0.6mL/min, detection at 300 nm and ambient temperature.

concentration in the mobile phase. The protein phase can undergo conformational changes at various conditions and hence, can effect separation of the analytes. The retention mechanism of the avidin column is mainly due to hydrophobic and electrostatic interactions. In our hands, the stability of the protein column was more than satisfactory. During the most recent six month period, retention times, separation factors, and resolution of enantiomers of thalidomide did not change significantly.

CONCLUSION

An isocratic HPLC-UV method was developed to quantitate thalidomide enantiomers in plasma using a new and unexplored avidin protein column. The solid phase extraction method for thalidomide is less labor intensive and the

chromatographic run time is less than 13 min. The method showed good accuracy and is sensitive and reproducible. Two of the commercially available metabolites of thalidomide, N-phthaloyl L-glutamic acid and phthalic acid were shown not to interfere with the assay method.

This method should be useful in the study of toxicokinetics and pharmacokinetics of thalidomide enantiomers in humans.

REFERENCES

1. R. L. Barnhill, A. C. McDougall, J. Am. Acad. Dermatol., 7, 317-323 (1982).

2. G. B. Vogelslang, S. Taylor, G. Gordon, Transplant Proc., **18**, 904-906, (1986).

3. S. Makonkawkeyoon, A. L. Moreira, Proc. Natl. Acad. Sci., USA , **90**, 5974-5978 (1993).

4. G. Blaschke, H. P. Kraft, Arzneimittelforschung, **29**,1640-1642 (1979).

5. S. Fabro, R. L. Smith, R. T. William, Nature (London), **215**, 296 (1967).

6. T. Eriksson, S. Bjorman, B. Roth, Chirality, 7, 44-52 (1995).

7. D. H. Sandberg, S. A. Bock, Anal. Biochem., **8**, 129-132 (1964).

8. T. Eriksson S. Bjorkman, H Ekberg; J. Chromatogr., **582**, 211-216 (1992).

9. T. L. Chen, G. B. Vogelsang, B. G. Petty; Drug Metab. Dispos., **17**, 402-405 (1988).

10. A. Delon, S. Favreliere, W. Couet, J. Liq. Chromatogr., **18**,279-309 (1995).

11. J. C. Reepmeyer, Chirality, **8**,11-17 (1996).

12. H. Aboul Enein, M. R. Islam, J. Liq. Chromatogr., **14**, 667-673 (1991).

13. B. Knoche, G. Blaschke, J. Chromatogr., **666**, 235-240 (1994).

14. Y. Oda, N. Asakawa, J. Chromatogr., **572**, 133-141 (1991).

15. M. J. Czejka, H. P. Koch, J. Chromatogr., **413**,181-187 (1987).

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