HPLC Determination of Tryptophan Enantiomers with Photometric, Fluorimetric and Diode-Laser Polarimetric Detection

F. García Sánchez* / A. Navas Díaz / A. García Pareja

Departamento de Química Analítica, Facultad de Ciencias, Universidad de Málaga, 29071 Málaga, Spain

Key Words

Column liquid chromatography
Tryptophan
Chiral separations
Diode-laser polarimetric detection

Summary

The combined utilization of photometric, fluorimetric and polarimetric detection liquid chromatography detectors in series for the identification and quantification of D-tryptophan and L-tryptophan was evaluated. Detection limits of about 1 μg were established with the range of linearity extending to about 100 μg. The relative standard deviation of the D-form and L-form tryptophan were 7.32 and 4.22 %, respectively. The amount unknown of tryptophan enantiomers in the different mixtures was determined with an accuracy of 1.58% at the 40 μg injection level.

Introduction

The resolution of enantiomers has traditionally been considered one of the more difficult problems in separations science. Enantiomers have identical physical and chemical properties in an isotropic environment except that they rotate the plane of polarized light in opposite directions. A mixture containing equal amounts of enantiomers is referred to as a racemic mixture. Neither racemic mixtures nor solutions of achiral compounds are able to rotate the plane of polarized light [1].

A significant proportion (approximately 25 %) of the most widely prescribed drugs are sold as racemic mixtures. The individual isomers of such mixtures frequently differ in pharmacological or metabolic activities [2], and often only one isomer is therapeutically active. L-tryptophan is a nutrient. It serves as the precursor of the neurotransmitter serotonin and of the behaviorly-active trace-amine tryptamine, its metabolism is of especial importance in neurochemistry [3], while D-tryptophan is inactive (Figure 1).

Methods have been described previously for the determination of tryptophan in blood, plasma, urine and other physiological samples by liquid chromatography with fluorimetric and photometric detection without chiral separation [4, 5, 6]. In addition there are methods for the enantiomeric separation of tryptophan using a chiral stationary phase in liquid chromatography [7, 8, 9]. An alternative solution is to use one or several on-line detectors to eliminate the need for chromatographic resolution of the enantiomers.

Numerous chromatographic techniques and detectors have been advanced as a means of differentiation and to quantitate enantiomeric purity. Several polarimetric detectors have been developed and used in the determination of enantiomeric purity [10, 11, 12]. The performance characteristics of the on-line diode laser polarimeter have been described [12].

The basis of this work is the enantiomeric purity determination of tryptophan by liquid chromatography without chiral separation. This paper describes the enantiomeric purity determination of tryptophan using photometric, fluorimetric and diode-laser polarimeter detectors in series, without chiral separation. The linearity of the chiral / photometric detectors and chiral / fluorimetric detectors is also discussed.

Experimental

Chemicals

The L-tryptophan, D-tryptophan and D,L-tryptophan were obtained from Merck (Darmstadt, Germany). Methanol and water were of Lichrosolv gradient grade (Merck).

Stock standard solutions of form-D, form-L and racemic tryptophan (4 g L^{-1}) were prepared by dissolving the

0009-5893/96/05 494-05 $ 03.00/0

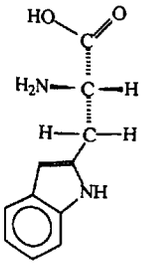
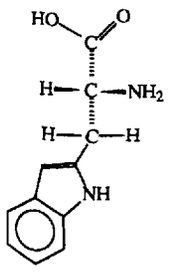
Structure	IUPAC Name	IUPAC abbreviature
	L-tryptophan	L-trp
	D-tryptophan	D-trp

Figure 1
Structures of the compounds studied.

compounds in hot methanol with protection from light. Working standard solutions were prepared by dilution with methanol. The solvents used as mobile phase were filtered through 0.2 μm nylon membrane filters and degassed.

Liquid Chromatography

The measurements were performed with a Merck-Hitachi (Darmstadt, Germany) Liquid Chromatograph consisting of a L-6200 pump, an AS-4000 autosampler, an L-4250 UV-visible detector, F-1080 fluorescence detector and a D-6000 interface. Integration was carried out with a PC/AT computer and instrument parameters were controlled by Hitachi-Merck HM software.

A ChiralMonitor 2000 optical rotation detector (Applied Chromatography Systems Limited, Macclesfield, England) placed in series with and after the UV-visible detector was equipped with a collimated laser diode providing upto 30 mW of light at 830 nm, and a flow cell of 0.48 dm path length, 73 μL volume. The polarimetric detection system has been described in detail elsewhere [11]. Because the analog output of the ChiralMonitor 2000 was not provided with software for managing data acquisition and processing, we implemented this complementary instrumentation. Data acquisition and transformation were accomplished by the Pico ADC-100 (Picotechnology Ltd., Cambridge, UK) which is an analog-to-digital converter with two input ranges. The instrumental parameters were controlled by Picolog Software (Picotechnology Ltd., Cambridge, UK) and the calculation of the areas (negative and positive peaks), the peaks heights and retention times were performed with Lab-Cal LC software (Galactic, Salem, NH). The Picolog program has been described in detail in elsewhere [13].

LC Operating Conditions

The tryptophan enantiomers were analyzed using a LiChrospher RP 8 reversed-phase column (25 cm × 4 mm I.D.; 10 μm particle size) from Merck (Darmstadt, Germany). The mobile phase composition was methanol/water (20:80 v/v) at 0.7 mL min^{-1} flow rate and detection was by means of photometric (λ = 280 nm), fluorimetric ($\lambda_{excitation}$ = 310 nm and $\lambda_{emission}$ = 440 nm) and polarimetric detectors.

Results and Discussion

Optimization of the Chromatographic Conditions

The signals from the polarimetric detector have a particular problem, namely the injection peak is very big and sensitive to variations in pressure due to pump pulsation, to thermal variation and to gas dissolved in the mobile phase. This effect can be avoided by retarding the elution of the analytical peak for two-three minutes after the injection peak. The analyte peaks must be separated from the injection peak, and the mobile phase must be previously well mixed and degassed. The selection of solvents for use in the mobile phase is doubly important because they influence the rotatory power of the analyte. The tryptophan enantiomers were separated from the injection peak with a mobile phase composition of methanol/water (20:80) at a flow rate of 0.7 mL min^{-1}.

Enantiomeric Determination Using Three Detectors in Series (UV, Fluorimeter and Polarimeter)

The linearity of response of the polarimetric detector has been investigated for tryptophan enantiomers. The least squares line for D-and L-tryptophan (trp) were (n = 5):

Area under peak = 1020 + 117 D-trp (μg) r = 0.9972

Area under peak = 3778 - 258 L-trp (μg) r = -0.9900

The chromatographic resolution of the enantiomers is poor, showing retention times of D-trp for the photometric, polarimetric and fluorimetric detectors of 9.18, 9.40 and 10.04 minutes, respectively. The relative standard deviations (n = 10) were 1.38, 1.31 and 1.18 %, respectively. The retention times of L-trp for the photometric, polarimetric and fluorimetric detectors were 9.40, 9.90 and 10.18 minutes, respectively. The relative standard deviations (n = 10) were 1.47, 1.30, and 1.40 %, respectively. Figure 2 shows chromatograms of the tryptophan enantiomers, D-trp and L-trp, corresponding to photometric, fluorimetric and polarimetric detection. The detection limits of D-trp and L-trp

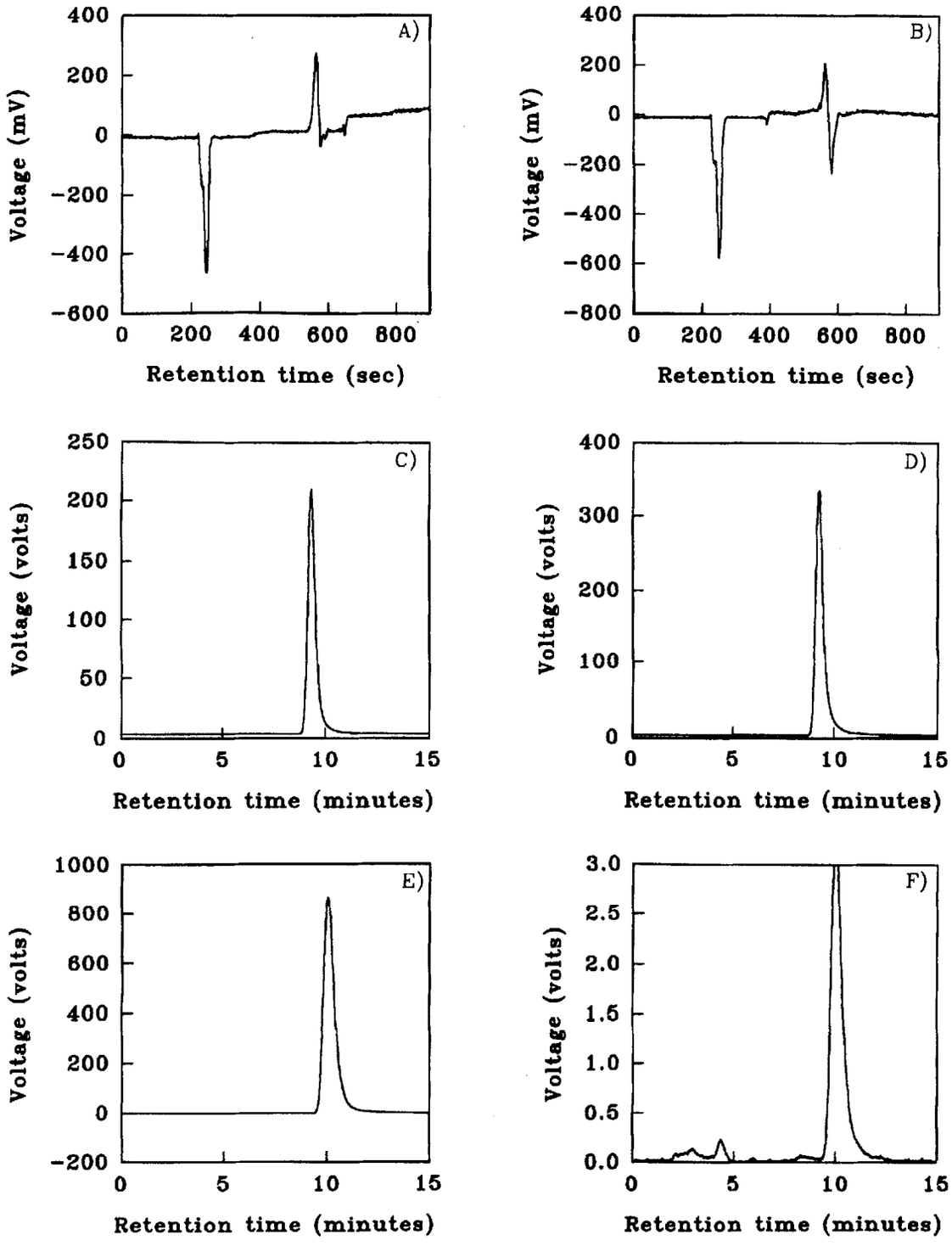
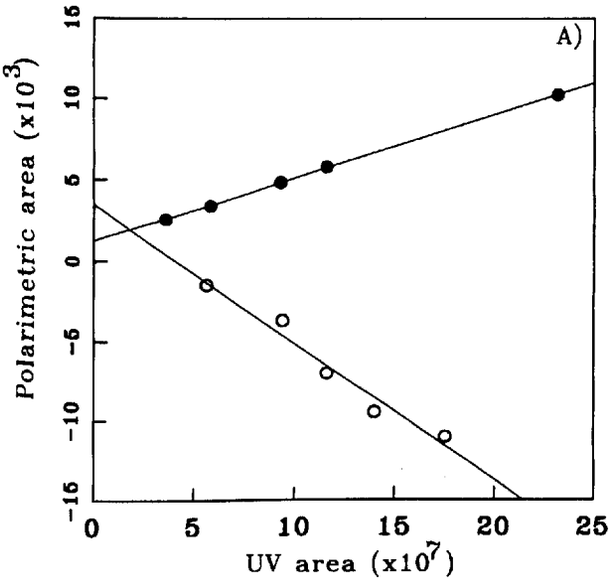


Figure 2
Chromatograms of D-Trp (A, C, E) and L-Trp (B, D, F) with polarimetric (A, B), photometric (C, D) and fluorimetric (E, F) detection. D-Trp = 20 µg; L-Trp = 30 µg.

in the polarimetric determination were 4.52 and 4.70 µg, respectively. Relative standard deviations (n = 6) were 7.32 and 4.22 % for D-trp and L-trp, respectively.

The linearity between detectors (UV, fluorimeter and polarimeter) has been investigated. For this we have considered the area of polarimetric signal vs the area of the UV (Figure 3A) and also vs the area of the fluorimeter (Figure 3B). Areas of polarimetric response were graphed against areas of UV response and areas of fluorescence response, eliminating any contribution to uncertainty from preparation of solutions or injection. Good correlations were obtained in both cases. The



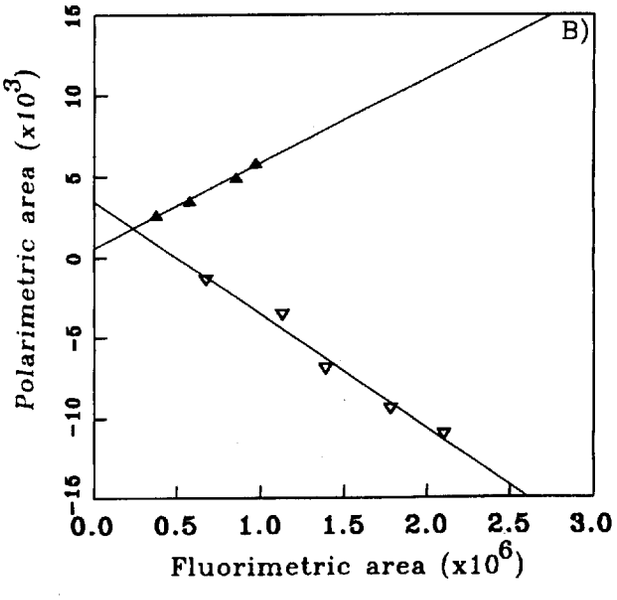
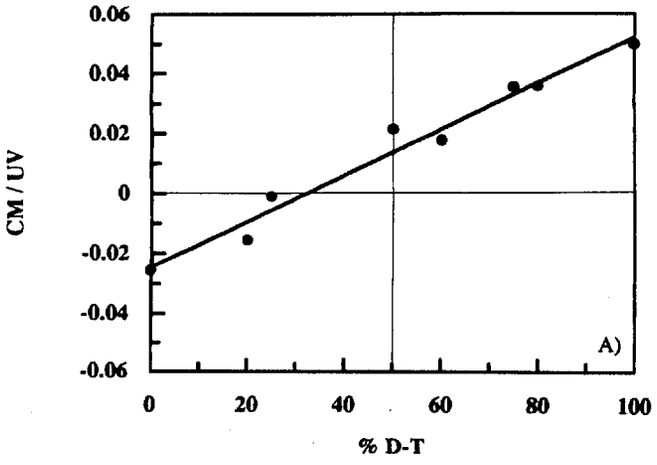


Figure 3
Response detector: A) polarimetric/photometric (● D-Trp, ○ L-Trp); B) polarimetric/fluorimetric (▲ D-Trp, Δ L-Trp). Range: D-Trp, 10–40 μg; L-Trp, 20–60 μg.



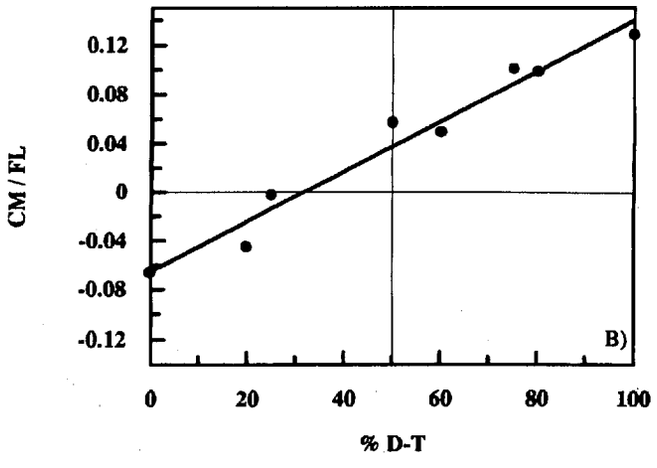


Figure 4
Area under peak ratio vs enantiomeric composition. A) polarimetric/photometric; B) polarimetric/fluorimetric. Total amount 40 μg.

magnitude of the slope of linearity plots of pure enantiomers should be identical while the signs should be opposite.

Various ratios of enantiomers were injected keeping the total amount constant at 40 μg. The results were plotted in Figure 4A as the ratio of the areas from the Chiral-Monitor and UV detectors vs the amount of D-trp in the sample and in Figure 4B as the area ratio of the Chiral-Monitor and fluorimetric detectors vs the amount of D-trp in the sample. The unknown amount of D- and L-tryptophan in the racemic mixtures was determined by measuring the area from each detector, ratioing those values and graphically determining the amount of the enantiomer present.

Table I shows the results obtained for a series of three synthetic samples of D- and L-trp calculated as in Figures 4A and 4B. The relative standard deviation of the measurements is less than 2 %, therefore it is deduced that the method is applicable to the determination of D- and L-trp in real samples.

A solution of D,L-trp, of unknown composition of the isomers was prepared and injected onto the chromatograph following the procedure previously described to determine the enantiomeric purity of a mixture of isomers. The results are shown in Table I.

The graphs in Figures 4A and 4B do not go through the point 50–0, that is to say, 50 % of D-trp and zero corresponding to the ordinate of the origin, which indicates that the isomer D-trp is not totally pure but contains small quantities of the isomer L-trp. To find the composition of L-trp in D-trp the theoretical graph is com-

Table I. Enantiomeric ratio determination of synthetic mixtures

%D-trp/%L-trp	CM / FL			CM / UV		
	%D-trp	%L-trp	%RSD	%D-trp	%L-trp %	%RSD
20 / 80	22.39	77.61	2.43	22.86	77.14	2.08
55 / 45	54.29	45.71	2.08	51.87	48.12	1.30
45 / 55	45.95	54.05	0.44	43.75	56.25	0.00
D,L-trp	40.95	59.05	2.42	39.86	60.14	1.89

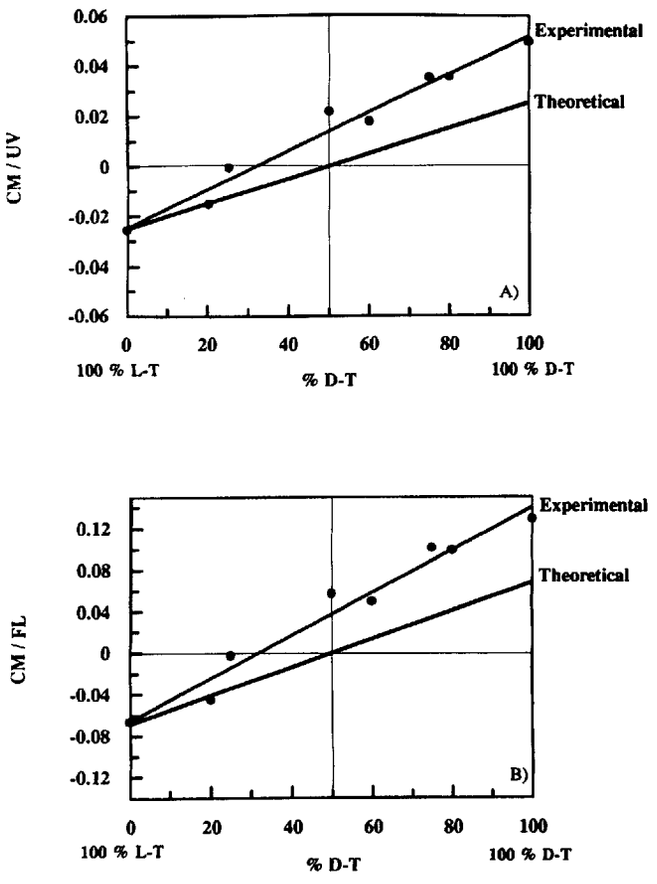


Figure 5
Area under peak ratio vs enantiomeric composition. Experimental and theoretical curves. A) polarimetric/photometric; B) polarimetric/fluorimetric.

pared with the experimental (Figure 5) which demonstrates a purity of 80 / 20 % in the commercially "pure" D-trp isomer.

Acknowledgements

We thank the Comisión Interministerial de Ciencia y Tecnología (Projects PB93 – 1006 and BIO94 – 0548).

References

[1] *D. W. Armstrong, S. M. Han*, Critical Reviews in Anal. Chem. **19**, 175 (1988).
[2] *J. W. Hubbard, D. Ganes, H. K. Lim, K. K. Midha*, Clin. Biochem. **19**, 107 (1986).
[3] *G. M. Anderson, W. C. Purdy*, Anal. Chem. **51**, 283 (1979).
[4] *I. Morita, M. Kawamoto, M. Hattori, K. Eguchi, K. Sekiba, H. Yoshida*, J. Chromatogr. **526**, 367 (1990).
[5] *M. B. Berardino, F. C. Roingeard, N. K. Fukagawa*, J. Nutr. Biochem. **1**, 220 (1990).
[6] *C. P. Bearcroft, M. J. G Farthing, D. Perret*, Biomed. Chromatogr. **9**, 23 (1995).
[7] *K. Uchikura, M. Kirisawa*, Anal. Sci. **7**, 971 (1991).
[8] *L. Soltes, B. Sebille, N. Thuaud*, Chromatographia **38**, 761 (1994).
[9] *S. C. Jacobson, G. Guiochon*, Anal. Chem. **64**, 1496 (1992).
[10] *W. Boehme, G. Wagner*, U. Oehme, U. Priesnitz, Anal. Chem. **54**, 709 (1982).
[11] *E. S. Yeung, L. E. Steenhoek, S. D. Woodruff, J. C. Kuo*, Anal. Chem. 52, 1399 (1980).
[12] *D. K. Lloyd, D. M. Goodall, H. Scrivener*, Anal. Chem. **61**, 1238 (1989).
[13] *A. Navas Díaz, F. Garcia Sanchez, A. Aguilar Gallardo, A. Garcia Pareja*, Instrument. Science Technology, **24**, 47 (1996).

Received: Dec 19, 1996
Revised manuscript received: Feb 22, 1996
Accepted: Mar 12, 1996