ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
26 (2001) 837–848

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

# Determination of optical purity by nonenantioselective LC using CD detection

Elena Bossu', Viviana Cotichini, Gianluca Gostoli, Anna Farina *

*Laboratorio di Chimica del Farmaco, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Rome, Italy*

Received 28 December 2000; received in revised form 4 April 2001; accepted 15 April 2001

**Abstract**

The use of a circular dichroism (CD) based HPLC detection system was recently described by some authors and proposed for a nonenantioselective HPLC enantiomeric purity determination. Indeed the system, measuring both CD and UV signals simultaneously, allows the evaluation of the *g* anisotropy factor. In order to experimentally support such an analytical procedure as an alternative to the enantioselective chromatographic method currently found in some pharmacopoeial monographs, we have studied its application to the analysis of dexchlorpheniramine maleate, an active substance which exhibits a poor CD signal in the 250–270 nm spectral region with a *g* value of the order of $10^{-4}$. The results reported indicate that the suitability of the studied procedure for the enantiomeric purity determination is obtained only when the CD-detector reaches high stability; indeed a certain time lag is systematically necessary to obtain stable responses, i.e. adequate precision. The enantioselective HPLC procedure seems to be more precise for enantiomeric purity values $\leq 2\%$ than the CD based detection system; such a disadvantage might be counterbalanced by the use of non chiral stationary phases. © 2001 Elsevier Science B.V. All rights reserved.

*Keywords:* Anisotropy factor (g); Chiroptical detector; Circular dichroism (CD); Enantiomeric purity (e.p.); HPLC

## 1. Introduction

The occurrence of single enantiomers of chiral drugs is significantly increasing in current marketed medicinal products. This tendency is reflected in the content of many pharmacopoeias, where quite a number of monographs are devoted to single enantiomers of active substances.

The determination of the enantiomeric purity (e.p.) by specific test, i.e. the content (%) of the active enantiomer, is a pharmacopoeial requirement for such drugs. Although enantioselective HPLC is the most commonly applied separation method, the last few years have seen the use of nonenantioselective HPLC methods, such as the application of nonenantioselective columns coupled with a dual circular dichroism (CD) absorption detector measuring CD and UV spectra simultaneously and providing, through their ratio, the anisotropy factor *g* [1–6].

The suitability of this nonenantioselective HPLC with a CD detector has been evaluated and

* Corresponding author. Tel.: +39-06-4990-2365; fax: +39-06-4938-7151.

*E-mail address:* chimorg@iss.it (A. Farina).

0731-7085/01/$ - see front matter © 2001 Elsevier Science B.V. All rights reserved.

PII: S0731-7085(01)00483-6

its use recommended in preference to enantioselective HPLC, when the control of enantiomeric purity is required at levels less than 99% [7].

Recently [8], the use of nonenantioselective HPLC with a CD detector has been also described for the assay of substances whose enantiomeric purity is better than 99% and even for compounds whose anisotropy factors are low ($g \cong 2 \times 10^{-4}$).

The recent introduction of CD as a pharmacopoeial method of analysis allows consideration of the applicability of the nonenantioselective chromatographic approach coupled with a CD detector, as an alternative to the enantioselective chromatographic methods presently found, in pharmacopoeial monographs, for the e.p. determination.

However, it must be shown that the new procedure is suitable for its intended purposes; in particular if a new detector system is used, adequate attention has to be paid to its contribution to the precision of the entire analytical procedure at each of the three levels: repeatability, intermediate precision and reproducibility [9].

The recently commercialized CD detector (Jasco mod. 1595) has been used in a relatively small number of LC methods and therefore, our attention has been focused on the study of specific issues like:

- the performance of the CD detector, particularly its repeatability,
- the reliability of the $g$ measurements when the CD spectra to be measured with the CD detector are of low intensity, i.e. when the experimental conditions for the determination of the anisotropy factor seem to be unfavourable.

A comparison of $g$ values obtained with two different procedures and instrumentations (i.e. $g$ values from measurements with a Jasco 720 spectropolarimeter and $g$ values from measurements with a Jasco 1595 CD detector in a chromatographic system) has been made for (+)-(*S*)-α-phenylglycine; such compound, whose enantiomeric purity was recently determined with a Jasco 720 spectropolarimeter at 218 nm [10], has spectral properties in the 240–270 nm range similar to those of some substances of pharmacopoeial interest, such as dexchlorpheniramine.

Subsequently dexchlorpheniramine maleate, whose Cotton effect is relatively small and whose monograph in the European Pharmacopoeia [11] presents a limit of 2% for the *R*-enantiomer, has been examined.

## 2. Experimental

### 2.1. Chemicals

(+)-(*S*)-α-phenylglycine, (*S*)-**1**, and (−)-(*R*)-α-phenylglycine, (*R*)-**1**, were commercial samples from Fluka (Buchs, Switzerland) and used without further purification. Dexchlorpheniramine maleate, (*S*)-**2**, or (+)-(*S*)-3-(4-chlorophenyl)-3-(pyridin-2-yl)-*N*,*N*-dimethylpropan-1-amine (Z)-butenedioate, and *rac*-chlorpheniramine maleate, *rac*-**2**, were pharmacopoeial chemical reference standards (Eur. Ph. Strasbourg, France). Their e.p., determined by HPLC on chiral stationary phases as described in the chromatographic methods section, resulted respectively as follows: (*S*)-**1** 99.11%, (*R*)-**1** 99.41% and (*S*)-**2** 99.58%.

Analytical grade sodium dihydrogen phosphate, potassium dihydrogen phosphate, phosphoric acid and sodium hydroxide were from Merck (Darmstadt, Germany). All solvents were HPLC grade (Carlo Erba, Milano, Italy); mobile phases prepared with buffer and organic solvents were filtered, before use, through a 0.45 μm filter.

### 2.2. Standards and working standard solutions

#### 2.2.1. Phenylglycine

A stock solution of (*S*)-**1** (5 mM) in sodium phosphate buffer (pH 3.0; 25mM) was diluted in the same solvent to obtain solutions in the dilution range of 1.97–5.00 mM (*spectropolarimetric measurements*).

A stock solution of (*S*)-**1** (10 mM) in sodium phosphate buffer (pH 3.0; 25mM) was diluted in the same solvent to obtain solutions in the dilution range of 5.00–10.00 mM (*nonenantioselective HPLC-CD detector measurements*).

Working standard solutions of (*S*)-**1** contaminated with known amounts of (*R*)-**1** were used for limit of detectability (LOD), limit of quantifica-

tion (LOQ) and e.p. determinations. An amount of (*R*)-**1** (equivalent to 4.61% w/w) was added to the stock solution of (*S*)-**1** (7.00 mM). The obtained solution was mixed in various proportions with the stock solution of (*S*)-**1** (7.00 mM) to obtain contaminated solutions containing the (*R*)-enantiomer ranging between 0.5 and 4.6% (w/w). These solutions were used in the HPLC experiments and, after appropriate dilution, in the spectropolarimetric measurements.

#### 2.2.2. Chlorpheniramine

Working standard solutions of (*S*)-2 in the concentration range of 1.06–1.78 mM (mobile phase as solvent), were prepared from a 1.78 mM stock solution in the same solvent (*nonenantioselective HPLC-CD detector measurements*).

LOD, LOQ [12] and e.p. determinations were performed using working standard solutions of (*S*)-**2** contaminated with known amounts of *rac*-**2**. A stock solution of (*S*)-**2** (1.49 mM) was mixed in various proportions with a stock solution of *rac*-**2** (1.50 mM) to have the (*R*)-enantiomer concentration in the range between 0.4 and 5.5% (w/w).

### 2.3. Instrumentation

Chromatographic analyses were performed using a Waters 600E pump (Mildford, MA) equipped with a Waters 717 plus autosampler, a Violet T55S column thermostat, a Waters 996 Photodiode Array (PDA) and a Jasco CD model 1595 (Tokyo, Japan) as detectors. The CD-1595

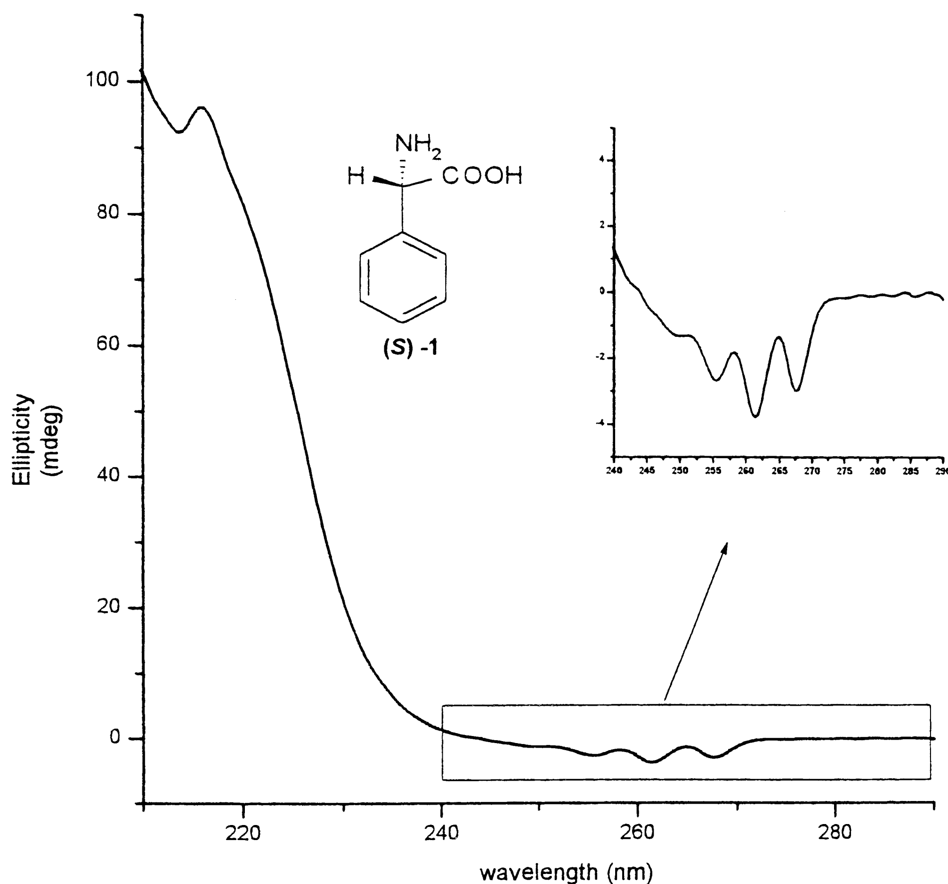


Fig. 1. CD spectrum of a solution of (*S*)-**1** (0.25 mM) in sodium phosphate buffer (pH 3.0; 50mM).

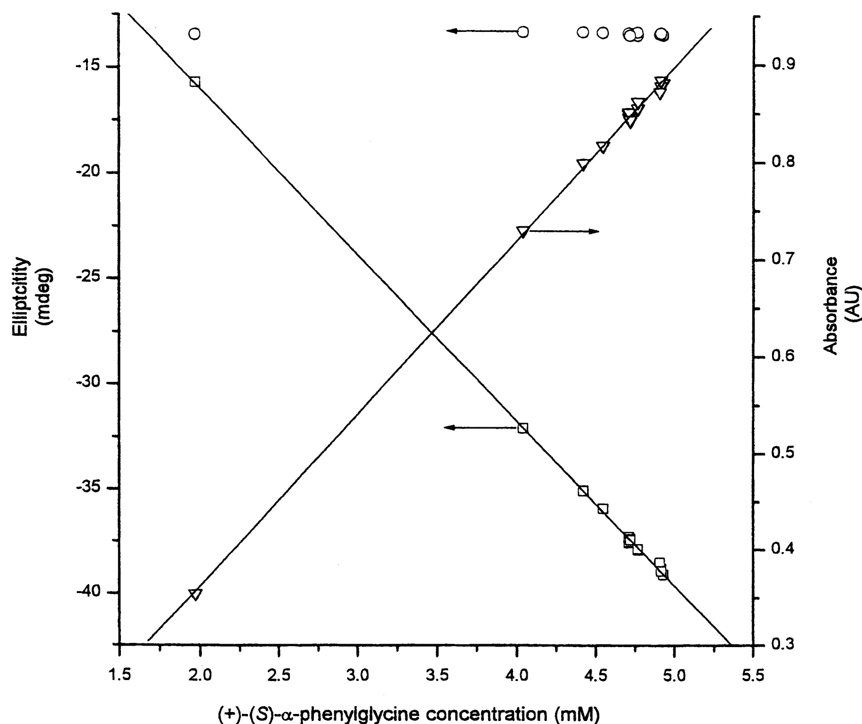


Fig. 2. Measured ellipticity (□), absorbance (∇) and calculated $g$ value $\times 10^{-5}$ (○) versus ($S$)-**1** mM concentration ($C_{S\text{-enantiomer}}$), sodium phosphate buffer (pH 3.0; 50mM) ($t = +20°C$). Best fit: $\theta = -0.189 - 7.866 \times C_{S\text{-enantiomer}}$; $r = 0.9997$ (left axis). $A = -0.004 + 0.178 \times C_{S\text{-enantiomer}}$; $r = 0.9994$ (right axis). Mean of $g = -1.337 \times 10^{-3} \pm 0.007 \times 10^{-3}$.

model performs simultaneous UV and CD detection and through their ratio, the evaluation of the $g$ factor. A Millennium 32 (Waters Chromatography) was used for acquisition and elaboration of the chromatographic data.

Spectropolarimetric measurements were carried out on a Jasco J-720 (Tokyo, Japan) spectropolarimeter (dichrograph) equipped with a PTC-348WI Jasco Peltier type temperature control.

### *2.4. Chromatographic methods*

#### *2.4.1. Enantioselective HPLC*

The e.p. determination of phenylglycine enantiomers was carried out using as chiral stationary phase a Chirobiotic T column (250 × 4.6 mm ID, 5 μm particle size), purchased from Advanced Separation Technologies Inc (Whippany, NJ), and as mobile phase water–ethanol (90:10) at a flow rate of 1 ml/min. The analyses were performed at 35 °C; UV and CD detection at 261 nm.

The e.p. determination of ($S$)-**2** was carried out as described in the dexchlorpheniramine maleate european monograph [11].

#### *2.4.2. Nonenantioselective HPLC*

Phenylglycine measurements were performed using a RP8 (Pinnacle octyl) column (150 × 4.6 mm ID, 5 μm particle size), purchased from Restek Corporation (Bellefonte, PA) and as mobile phase a 50 mM sodium phosphate buffer pH 3.0 at a flow rate of 1 ml/min; the column temperature was maintained at 20 °C. The wavelength utilised for the CD detection was 261 nm, while the PDA conditions were: spectral range from 200 to 320 nm; sampling rate 1.0; resolution 1.2 nm.

Dexchlorpheniramine measurements were performed using a Supelcosil ABZ-plus column (250 × 4.6 mm ID, 5 μm particle size), purchased from Supelco (Bellefonte, PA) and, as mobile phase, a mixture of 25 mM potassium phosphate

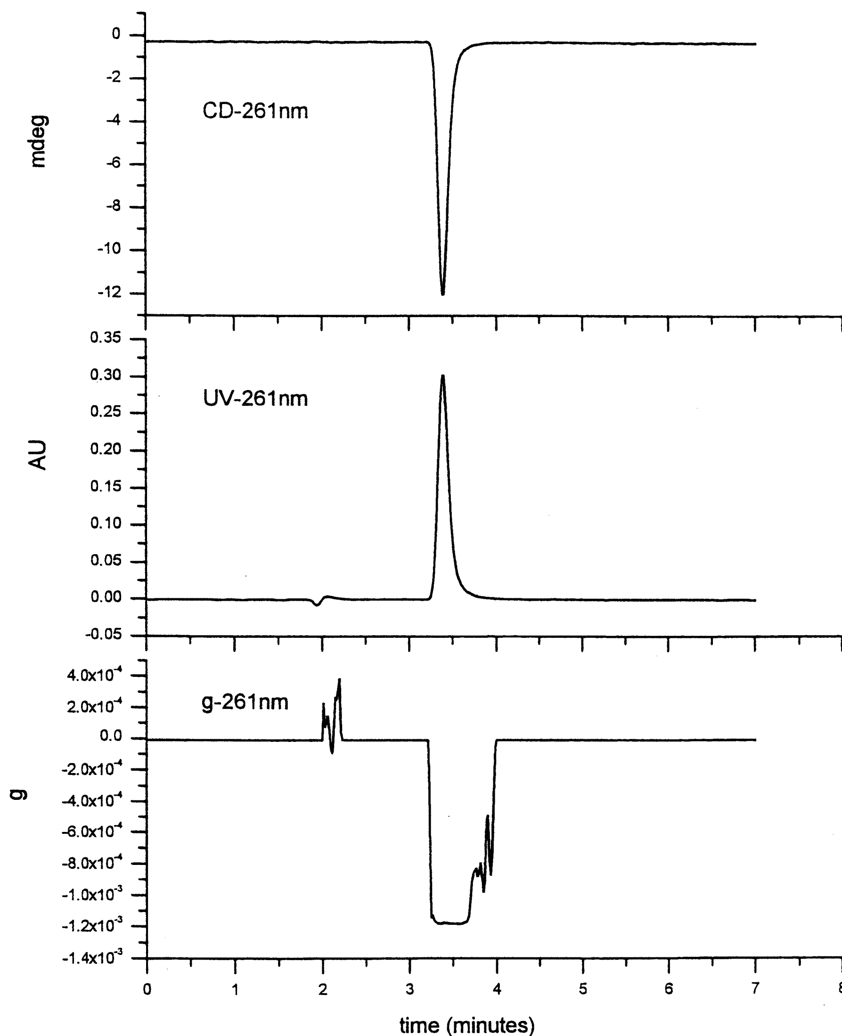


Fig. 3. CD, UV and anisotropy factor monitoring of (*S*)-**1** (7.025 mM); injected volume 20 μl. Chromatographic conditions: see experimental nonenantioselective HPLC.

(pH 7.0; 25 mM)–acetonitrile (60:40, v/v), at a flow rate of 1 ml/min. The CD detection was performed at 264 nm while the PDA conditions were the same as utilised in the analysis of phenylglycine.

*2.4.3. Spectropolarimetric method*

CD spectra with the Jasco 720 spectropolarimeter were recorded in wavelength mode from 200 to 320 nm, in the following conditions: cell length 1 cm, resolution 0.2 nm, band width 2 nm, response 2 s and scan speed 20 nm/s.

The ellipticity and the absorbance values were recorded in 'time mode' using the following parameters: resolution 1 nm, band width 2 nm, response 1 s, accumulations 3 × 60 s.

## 3. Result and discussion

### *3.1. Phenylglycine*

#### *3.1.1. Spectropolarimetric measurements*

The CD spectrum of (*S*)-**1** in phoshate buffer (pH 3.0) is shown in Fig. 1; it practically overlaps

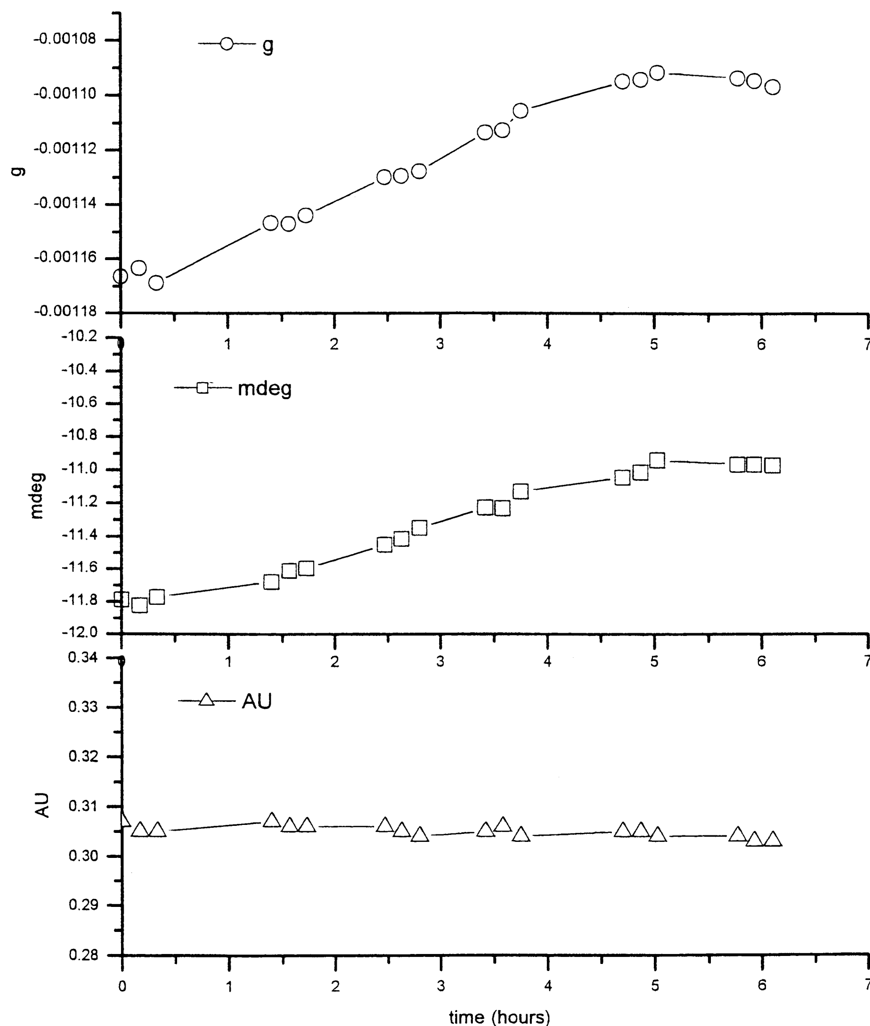


Fig. 4. Anisotropy factor, CD, and UV measurements of (*S*)-**1** (7.025 mM) by HPLC-CD detector as a function of time. Injected volume 20 μl. Chromatographic conditions: see experimental nonenantioselective HPLC.

the reported [10] spectrum of the substance dissolved in HCl (1M). The spectral region around 220 nm, where strong CD and UV signals are observed, was previously [10] used for ellipticity and absorbance measurements; our attention was focused on the less favourable spectral region (250–270 nm) where a lower signal is present; poor spectral properties are indeed common to chiral substances described in the Eur.Ph.

Ellipticity ($\theta$), absorbance ($A$) and anisotropy factor ($g$) values from measurements at 261 nm ($t = +20$ °C) are presented in Fig. 2 versus (*S*)-**1** concentration; as expected, $g$ is concentration independent.

The g factor of (*R*)-**1** resulted in an identical value with opposite sign.

(*R*)- and (*S*)-phenylglycine stock solutions were mixed as required (see experimental) in order to generate the calibration plot ($t = +20$ °C); the corresponding equation is

$$g = -1.370 \times 10^{-3} + 0.032 \times 10^{-3} \times C\%_{R\text{-enantiomer}}.$$

LOD and LOQ, respectively calculated [12] from $3.3\sigma/S$ and $10\ \sigma/S$, where $\sigma$ is the standard

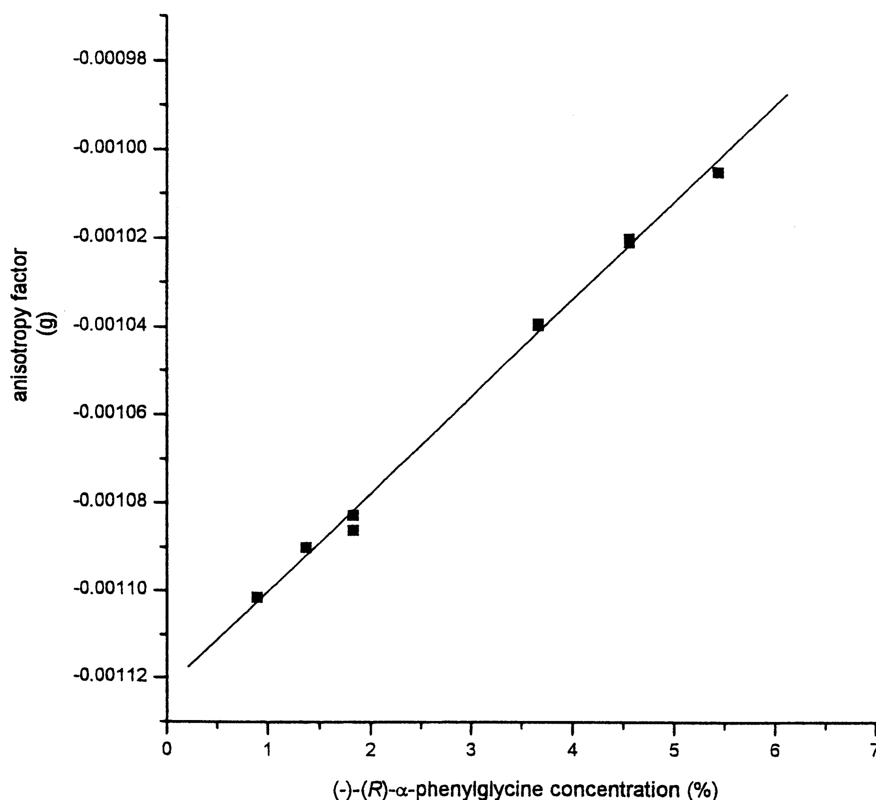


Fig. 5. Anisotropy factor values of ($S$)-**1** from HPLC-CD detector measurements versus $C\%$ of ($R$)-**1** ($C\%_{R\text{-enantiomer}}$). Curve equation: $g = -1.120 \times 10^{-3} + 0.022 \times 10^{-3} C\%_{R\text{-enantiomer}}$; $r = 0.9980$.

deviation of the response and $S$ the slope of the calibration curve, are shown in Table 1.

### 3.1.2. *Chromatographic measurements with CD detector*

Chromatograms displaying UV/CD responses and the anisotropy factor ($g$) trace of ($S$)-**1**, monitored at 261 nm, are shown in Fig. 3. The wavelenght of monitoring is practically settled by the CD spectrum of ($S$)-1 (see Fig. 1) in the 250–270 nm spectral range and by the band width (20 nm) of the CD detector itself.

Measurements have been repeated as a function of time. The results obtained (Fig. 4), which consistently overlap in interday experiments, show an initial decrease of the absolute value of the CD signal and of the anisotropy factor followed by a substantial constant and stable response.

Baseline instability has been excluded as a possible cause of the decrease of CD and $g$ factor signals; consequently the observed trend of the measured parameters might be related to the CD detector itself when used in analysing compounds whose CD spectra are quite poor.

We calculated the anisotropy factor from the ratio CD/UV in terms of peak height in the chromatograms (the quite narrow UV and CD signals allowed the peak height use); this numeri-

Table 1
Estimated LOD and LOQ of (−)-(R)-α-phenylglycine in (+)-($S$)-α-phenylglycine from measurements with a CD spectropolarimeter and a HPLC-CD detector

| | LOD (%) | LOQ (%) |
|---|---|---|
| CD spectropolarimeter | 0.27 | 0.82 |
| CD detector | 0.33 | $1.10 \pm 0.07$ |

cal value is comparable with that obtained by spectropolarimetric measurements (see Table 2).

To verify the reliability of the HPLC-CD detector procedure for the enantiomeric purity determination of (*S*)-**1**, measurements were performed in the time range of stable CD signals. The plot presented in Fig. 5 was obtained from measurements with appropriate solutions (see Section 2). The corresponding calibration equation is

$$g = -1.120 \times 10^{-3} + 0.022 \times 10^{-3} \times C\%_{R\text{-enantiomer}}.$$

Estimated LOD and LOQ numerical values are shown in Table 1.

### 3.2. Chlorpheniramine

#### 3.2.1. Chromatographic measurements with CD detector

CD spectra of (*S*)-**2** in various solvents are shown in Fig. 6. The intensity and sign of the broad band around 265 nm is dependent on the solvent nature; such dependence points out that the observed CD signal seems to be strongly related to the $n \rightarrow \pi^*$ transition of the chirally perturbed pyridine chromophore. Indeed in apolar solvents the lone pair of the pyridine nitrogen is relatively unperturbed while hydrogen-bond formation, taking place in polar solvents, might be responsible for the observed intensity changes

Table 2
(+)-(*S*)-α-phenylglycine *g* factor ($n = 15$) at 261 nm and relative standard deviation (RSD)

| | *G* | RSD |
|---|---|---|
| [a] | $-1.337 \times 10^{-3} \pm 0.007 \times 10^{-3}$ | 0.56 |
| [b] | $-1.102 \times 10^{-3} \pm 0.007 \times 10^{-3}$ | 0.68 |

[a] From spectropolarimeter.
[b] From HPLC-CD detector as CD/UV ratio (peak height).

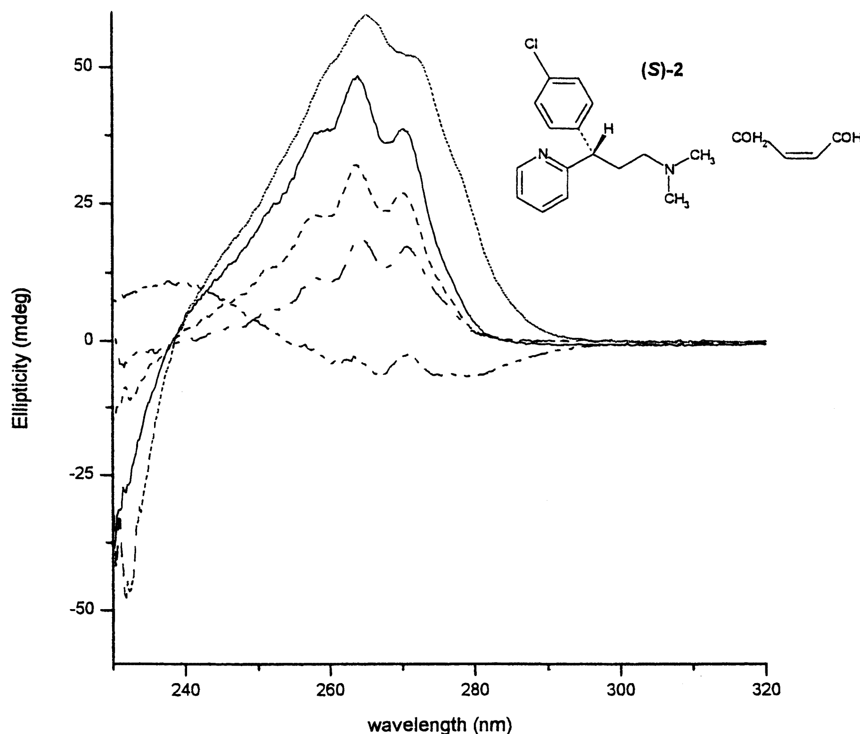


Fig. 6. CD spectra of (*S*)-**2** (0.31mM) in various solvents. Solvents: hexane-2-propanol-diethylamine (98:2:0.3, v/v/v) (-----); sodium phosphate buffer (pH 7.0; 25mM)-acetonitrile (60:40, v/v) ( — ); water (– – –);sodium phosphate buffer (pH 10.5; 50mM)-acetonitrile (50:50, v/v) (–––––); sodium phosphate buffer (pH 3.0; 50 mM)- acetonitrile (75:25, v/v) (– -- – -- –)

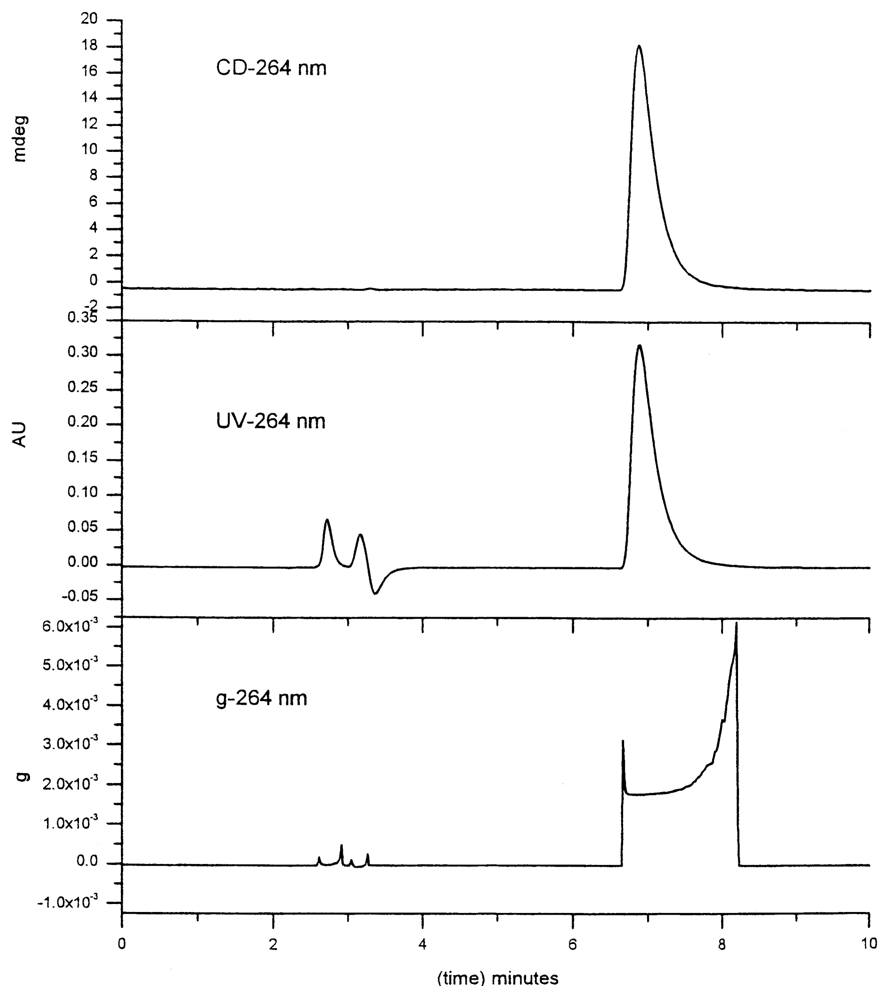


Fig. 7. CD, UV and anisotropy factor monitoring of (*S*)-**2** (1.46 mM); injected volume 10 μl. Chromatographic conditions: see experimental enantioselective HPLC.

as a consequence of the blue-shift of the $n \rightarrow \pi^*$ transition itself. When protonation of the pyridine ring occurs, the $n \rightarrow \pi^*$ transition is no longer present and a sign inversion is observed in the CD signal. A study of the CD spectra of chiral pyridine-substituted crown ether [13] suggests that the pyridine $n \rightarrow \pi^*$ transition is a sensitive probe of the nature of interacting chemical moieties.

Acetonitrile with sodium phosphate buffer (pH 7.0; 25 mM) has been chosen as the most favourable solvent for the HPLC-CD detector measurements of the enantiomeric purity of dexchlorpheniramine; Fig. 7 shows the chromatograms displaying UV/CD responses and the *g* factor trace monitored at 264 nm. An initial time dependence of the CD signal was observed in analogy to the (*S*)-**1** experiments. Consequently the reported measurements were performed when the detector stability was achieved; in such experimental conditions the repeatibility of the *g* factor value, calculated from the ratio CD/UV signals in terms of peak area (the broad UV and CD signals require the peak area use), was indeed satisfactory (intra-day RSD%: 0.4% ÷ 0.7%; inter-day RSD%:0.5% ÷ 0.9%).

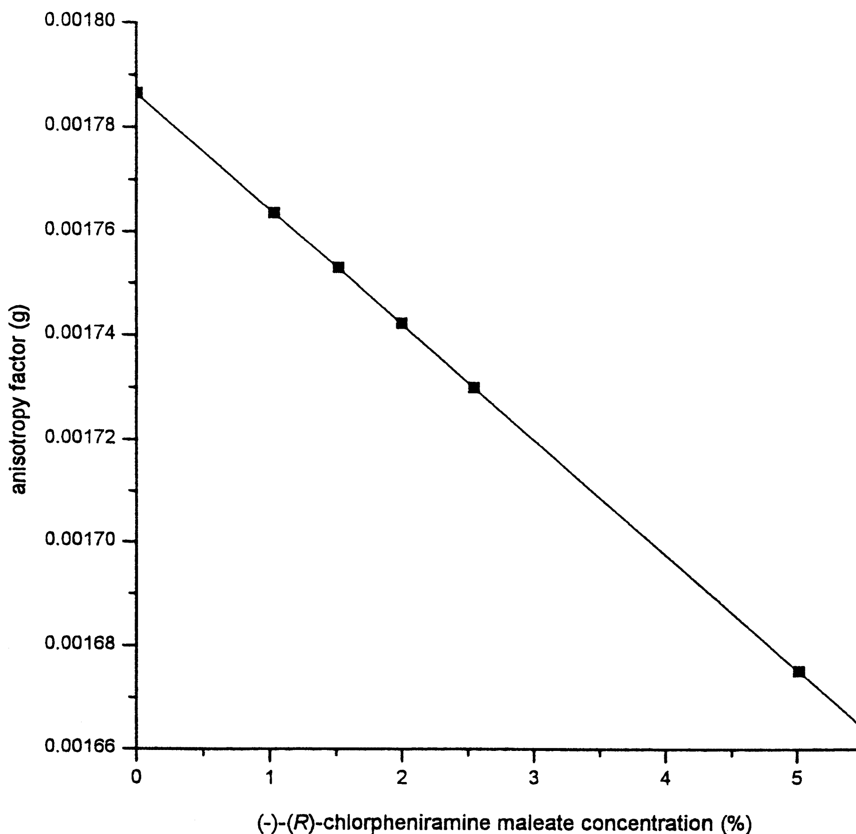


Fig. 8. Anisotropy factor of ($S$)-**2** from HPLC-CD detector measurements versus $C\%$ of ($R$)-enantiomer of chlorpheniramine ($C\%_{R\text{-enantiomer}}$). Curve equation: $g = 1.789 \times 10^{-3} - 0.022 \times 10^{-3} \times C\%_{R\text{-enantiomer}}$; $r = 0.9996$.

Table 3
Intra-day precision and accuracy for the $R$-enantiomer of chlorpheniramine maleate obtained by spiking solutions of known dexchlorpheniramine maleate concentration

| $R$-enantiomer% amount spiked[a] | day | Achiral HPLC-CD ($n=6$) | | | Chiral HPLC ($n=4$) | | |
|---|---|---|---|---|---|---|---|
| | | Mean | RSD(%) | Recovery (%) | Mean | RSD (%) | Recovery (%) |
| 1.45 | 1 | 1.48 | 12.4 | 101.4 | 1.44 | 4.6 | 99.3 |
| 1.46 | 2 | 1.47 | 16.7 | 101.0 | – | – | – |
| 1.46 | 3 | 1.42 | 14.6 | 97.2 | 1.47 | 6.1 | 101.0 |
| 2.99 | 1 | 2.97 | 5.7 | 99.3 | 3.02 | 1.9 | 101.0 |
| 3.00 | 2 | 2.98 | 7.0 | 99.3 | – | – | – |
| 3.02 | 3 | 2.98 | 10.7 | 98.7 | 2.98 | 2.0 | 98.7 |
| 5.55 | 1 | 5.62 | 5.1 | 101.2 | 5.70 | 4.0 | 102.7 |
| 5.58 | 2 | 5.61 | 4.5 | 100.5 | – | – | – |
| 5.61 | 3 | 5.63 | 4.9 | 100.4 | 5.53 | 1.5 | 98.6 |

[a] The $R$-enantiomer content in bulk dexchlorpheniramine maleate was 0.42%. The reported% spiked amounts represents the sum of 0.42 plus the added quantity. Freshly spiked solutions were prepared daily.

A calibration plot, referring to measurements at 264 nm (see Fig. 8) has been obtained using appropriate solutions (see Section 2) of known enantiomeric purity. The corresponding calibration equation is

$$g = 1.789 \times 10^{-3} - 0.222 \times 10^{-3} \times C\%_{R\text{-enantiomer}}.$$

A good linearity is shown with a correlation coefficient $r = 0.9996$. Agreement between interday experiments has been also observed ($r$ in the range 0.9995–0.9999).

Accuracy has been expressed as % recovery of added $R$-enantiomer in the range 1.5–5.5% (see Table 3), while the relative standard deviation (RSD) is presented in relation to the precision (see Table 3).

In order to compare such data with those relative to the enantioselective HPLC method [11] analysis of the same mixtures have been accomplished using the last procedure (see experimental); the obtained% recovery are shown in Table 3.

The accuracy of the two procedures is quite similar, the obtained recovery is around 100% for both methods; the precision of the chiral-HPLC seems to be better particularly when the amount of added $R$-enantiomer is less than 2%.

Estimated LOD and LOQ numerical values, respectively 0.18 and 0.54%, seem to be in agreement with the fact that the $g$ numerical value of dexchlorpheniramine is approximately two times that for the (+)-($S$)-α-phenylglycine.

## 4. Conclusions

A simple and rapid nonenantioselective HPLC method using a CD detector for the determination of the enantiomeric purity of dexchlorpheniramine maleate has been developed. Such a method, which does not require the enantiomer separation, seems to ensure adequate performance; indeed the results obtained and the reported data on precision, linearity and sensitivity show that the described nonenantioselective LC–CD system is suitable for determination of enantiomeric purity of compounds having an unfavourable CD signal.

The lower precision of the nonenantioselective HPLC approach with respect to the chiral HPLC procedure is balanced by the significant advantage of the use of nonchiral stationary phases and also in terms of fast method development.

## Acknowledgements

The authors thank Carmine De Sena for his valuable technical assistance.

## References

[1] A. Gergely, in: N. Purdie, H.B. Brittain (Eds.), Analytical Applications of Circular Dichroism, Elsevier Science, Amsterdam, 1994, pp. 279–292.

[2] C. Bertucci, E. Domenici, P. Salvadori, Circular dichroism detection in high-performance liquid chromatography: evaluation of the anisotropy factor, J. Pharm. Biomed. Anal. 8 (1990) 843–846.

[3] P. Salvadori, C. Bertucci, C. Rosini, Circular dichroism detection in HPLC, Chirality 3 (1991) 376–385.

[4] C. Bertucci, P. Salvadori, L.F. Lopes, J. Guimaraes, Determination of optical purity by high-performance liquid chromatography upon non-chiral stationary phases with dual circular dichroism/absorption detection, J. Chromatogr. Part A 666 (1994) 535–539.

[5] J. Zukowsky, Y. Tang, A. Berthod, D. Armstrong, Investigation of a circular dichroism spectrometer as a liquid chromatography detector for enantiomers: sensitivity, advantages and limitations, Anal. Chim. Acta 258 (1992) 83–92.

[6] K. Kudo, K. Ajima, M. Sakamoto, M. Saito, S. Morris, E. Castiglioni, New circular dichroism (CD) based detection for monitoring chiral compounds in HPLC, Chromatography 20 (1999) 59–64.

[7] M. Hadley, G. Jonas, An evaluation of the Jasco CD-995: a detector for the simultaneous measurement of chemical and enantiomeric purity, Enantiomer 5 (2000) 357–368.

[8] C. Bertucci, V. Andrisano, V. Cavrini, E. Castiglioni, Reliable assay of extreme enantiomeric purity values by a new circular dichroism based HPLC detection system, Chirality 12 (2000) 84–92 and references therein.

[9] European Pharmacopoeia, Technical Guide for the Elaboration of Monographs, 3rd ed., Pharmeuropa, special issue 1999.

[10] P. Horváth, A. Gergely, B. Noszál, Determination of enantiomeric purity by simultaneous dual circular dichroism and ultraviolet spectroscopy, Talanta 44 (1997) 1479–1485.

[11] European Pharmacopoeia 3rd Edition, Monograph 1196, Council of Europe, Strasbourg, France, supplement 2001, 709–711.

[12] Validation of analytical procedures: Methodology. ICH guidelines: CPMP/ICH/281/95.
[13] P.E. Schipper, R.M. Kellogg, Theoretical structure-spectrum relationships in the circular dichroism of chiral crown ethers: application to chiral pyridine-substituted crowns, J. Am. Chem. Soc. 112 (1990) 1471–1479.