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Determination of enantiomeric excess by chiral liquid chromatography without enantiomerically pure starting standards

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ABSTRACT: A facile approach for the enantiomeric excess determination of enantiomeric mixtures without the necessity of pure enantiomer standards is presented. Promethazine and trimeprazine commercial nonracemic mixtures were used as cases study to probe the validity of the method. Chromatographic resolutions obtained with a chiral column AGP in reverse phase mode were 1.32–1.16 (promethazine) and 1.20–0.93 (trimeprazine) for the three detectors (circular dichroism, photometric and fluorimetric) in series. Results obtained showed that enantiomeric excess was 10.4, 8.71 and 8.58% for promethazine and 1.60, 1.23 and 1.80% for trimeprazine (medium values of 9.23 ± 1.01% and 1.54 ± 0.29%, respectively). Recovery assay over human serum samples, at three concentration levels, spiked with prometazine and submitted to solid-phase extraction, gave values of 99.09–93.48% [*S*-(−) enantiomer] and 98.51–91.89% [*R*-(+)-enantiomer]. Detection limits of promethazine enantiomers were between 0.02 μg (fluorimetric) and 1 μg (circular dichroism), and 0.02–1.1 μg for trimeprazine. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: enantiomeric excess; approach without standards; circular dichroism; liquid chromatography; promethazine; trimeprazine

Introduction

Generally, the determination of enantiomeric excess (ee) of chiral compounds is performed by chiral HPLC (Mehta, 1998; Andersson *et al*., 2003; Matthijs *et al*., 2004) using nonspecific detectors such as UV or fluorescence (Sanchez *et al*., 1996). In other instances (Bertucci *et al*., 2000; Sanchez *et al*., 2008a) achiral HPLC coupled to chiroptical detectors allows quantification of ee by using the anisotropy factor (*g*) as an analytical signal (Reetz *et al*., 2000). However, qualitative identification of the enantiomers, elution order and quantitative determination must be supported by the availability of pure enantiomer standards. This has been pointed out recently (Wu *et al*., 1990; Sanchez *et al*., 2008a).

For analytical research, if no commercial pure enantiomers are available, one of two alternatives must be used: asymmetric synthesis and purification of the enantiomeric pair, or a gift from a pharmaceutical or chemical company. This is especially important in the research and discovery of new pharmaceutical products showing chirality.

The use of circular dichroism (CD) and optical rotation (OR) detectors in HPLC is restrained by the lack of sensitivity compared with UV/fluorescence detectors, in spite of their chiral specific character. In this study we investigate the accuracy and precision of the methodology for the ee determination of promethazine and trimeprazine racemic commercial products without the aid of pure enantiomer standards. The methodology is based on the use of chiral HPLC coupled to CD/OR detectors. Using this approach, every enantiomer mixture can be investigated and submitted to ee analysis without necessity of buying (if available) the pure enantiomers.

A bibliographic search showed that seven papers have been published dealing with the determination of enantiomeric excess in the absence of reference samples. Two of them deal with the enantiomer identification by HPLC/MS/CD of clarithomicin and rifamphicin (Oswald *et al*., 2011; Van der Elst *et al*., 2011). The inverted chirality approach for enantiomeric excess determination of camptothecin in the absence of reference standards was used by Badaloni *et al*. (2007, 2010). A further two used HPLC/MS/OR (Goss *et al*., 2000) for identification of trace impurities in chiral analysis, and HPLC/NMR/CD (Mistry *et al*., 1999) for enantiomeric structural identification and composition of atracurium besylate. In the last paper the authors (Wang *et al*., 2010) used an stereoselective enzymatic transformation of methadone and its chiral metabolite by citochrome P-450.

When a nonchiral detector (UV/Fl) detector is used, the response factor of both enantiomers must be the same. This is true because the physico-chemical properties of the enantiomers are identical in an isotropic environment. Only chiroptical detectors, which introduce an anisotropic electromagnetic environment, give different (bimodal) response factor, at least in sign.

Using as a standard the commercial product (racemic or not), provided it has adequate purity, if the chromatographic

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Abbreviations used: CD, circular dichroism; OR, and optical rotation; PMT, promethazine; TMP, trimeprazine

separation is performed with a reasonable index of selectivity, the chiroptical OR/CD detector gives the character *R* or *S* of the eluted enantiomers, and UV/Fl or OR/CD allows calibration functions of both enantiomers to be performed.

In this work a commercial promethazine (PMT) mixture of enantiomers and a commercial trimeprazine (TMP) mixture of enantiomers (Zayas *et al.*, 1999) were used as case study to prove the analytical performance of the methodology (Figure 1). Commercial products containing PMT in the USA are named Pentazine and Phenergan, and products containing trimeprazine are named Allergan and Alimezine. Pharmacological uses are mainly as antihistaminic and sedative-hypnotic.

Experimental

Reagents and materials.

Promethazine hydrochloride was supplied by Sigma (St Louis, MO, USA) and recrystallized from ethanol–water (90:10). Trimeprazine hemitartrate was purchased in Sigma and recrystallized twice from methanol–water (50:50). Lyophilized human serum was obtained from Sigma (ref. H1-388). A chiral AGP (Chrom Tech., Congleton, UK) column was utilized (125 × 4.6 mm, 5 μm particle size). High-purity deionized water was obtained from a Milli-Q water purification system (Millipore, Canada). All solvents used were gradient-grade Lichrosolv UV–vis (Merck, Darmstadt, Germany). Phosphate buffer saline (PBS) was prepared from analytical reagent sodium phosphate and HCl (Merck). Standard promethazine and trimeprazine solutions were prepared by dissolving 50 mg in 50 ml ethanol, stored at 4°C in the absence of light. Working solutions were then prepared by dilution with the appropriate mobile phase.

Instrumentation

The measurements were performed with a Jasco (Tokyo, Japan) liquid chromatograph equipped with a Degassy Popular DP4003, Jasco intelligent pump model PU-1580, Jasco LG-1580-04 quaternary gradient unit, Jasco intelligent auto sampler model AS-2055 Plus with a 100 ml sample loop, Jasco interface modulated LC-NetII/ADC, chiral CD detector Jasco CD-2095 equipped with an Hg–Xe lamp (150 W) and a Gland-Taylor polarizer prism, and Jasco OR-2090 polarimetric detector. A standard tapered flow cell of 25 mm path length and a Monk–Gillieson mounting monochromator were used. Fluorescence signals were recovered from a F-1080 fluorescence detector (Merck-Hitachi). A Lichrolut® SPE extraction and drying unit (Merck, Darmstadt, Germany) was used.

Chromatographic conditions

Promethazine. A 250 nm wavelength was used in all measurements (CD and UV detectors) and $\lambda_{exc} = 250$ nm, $\lambda_{em} = 340$ nm (fluorescence detector). A mobile phase 20 mM PBS pH4.15 at a flow-rate of 0.8 mL/min was used, isocratic mode. The injection volume was 10 μL. Sample dilutions were in the mobile phase.

Trimeprazine. A 256 nm wavelength was used in all measurements (CD and UV) and $\lambda_{exc} = 290$ nm, $\lambda_{em} = 445$ nm (fluorescence detector). The mobile phase was 20 mM PBS pH 3.31 at a flow-rate of 0.6 mL/min in isocratic mode. The injection volume was 10 μL, diluted in mobile phase.

Solid-phase extraction

Solid-phase extraction (SPE) of samples were carried out on disposable C_{18} 200 mg 3 mL (40–63 μm) Lichrolut (Merck). The columns were conditioned with 1 mL of methanol followed by 1 mL of water. Following that, 1 mL of serum spiked with PMT was loaded. The columns were washed twice with 1 mL of water. Elution was carried out four times with 250 μL of 20 mM PBS, pH 4.15–isopropanol (99.5:0.5, v/v) and diluted to 1 mL with the mobile phase. Aliquots of this were injected into the HPLC system.

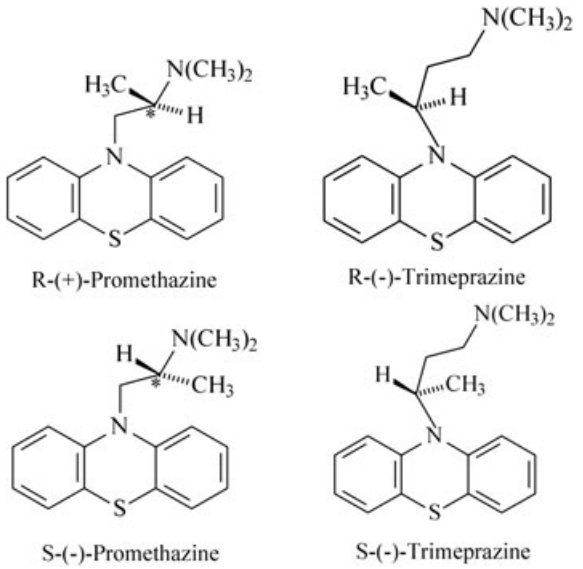

Figure 1. Structure of promethazine enantiomers and trimeprazine enantiomers.

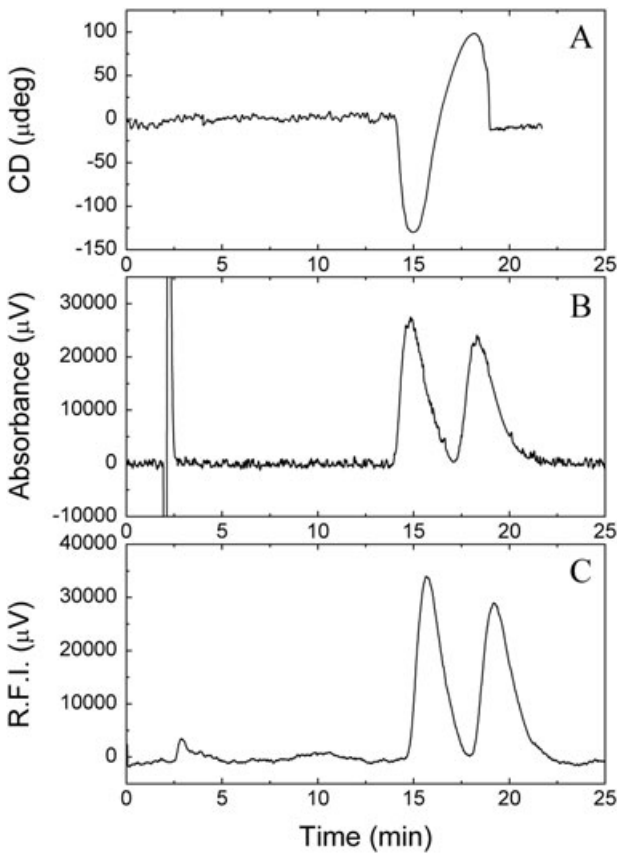

Figure 2. Chromatograms of promethazine. (A) Circular dichroism detector ($\lambda = 250$ nm). (B) UV detector ($\lambda = 250$ nm). (C) fluorescence detector ($\lambda_{exc} = 250$ nm, ($\lambda_{em} = 340$ nm). Chiral column AGP, mobile phase PBS, pH 4.15–isopropanol (99.5:0.5, v/v). Flow-rate 0.8 mL/min. $\lambda = 250$ nm. Injection 5 μg in 10 μL.

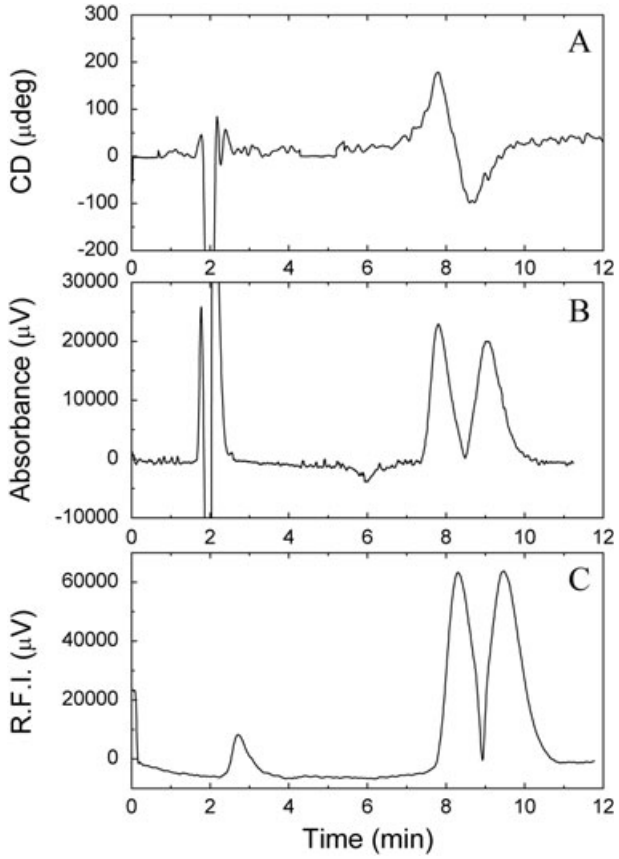

Figure 3. Chromatograms of trimeprazine. (A) Circular dichroism detector ($\lambda = 256$ nm). (B) UV detector ($\lambda = 256$ nm). (C) fluorescence detector ($\lambda_{exc} = 290$ nm, ($\lambda_{em} = 445$ nm). Chiral column AGP, mobile phase PBS pH 3.31–isopropanol (99.0:1.0, v/v). Flow-rate 0.6 mL/min. $\lambda = 256$ nm. Injection 5 μg in 10 μL.

Results and discussion

The proposed methodology is based on two requisites: good chromatographic separation of the enantiomers and good purity of the mixture of enantiomers used as standard. Provided this is accomplished and the response factors of the pair are identical, we can write

$$A = \varepsilon bc,\ A' = \varepsilon bc',\ \text{and}\ A/A' = c/c'$$

in which A and A' are the absorbances or areas of each chromatographic peak, ε the molar absorption coefficient, and c and c' are concentrations of each enantiomer in the mixture. If Cs is the total concentration of the standard, $Cs = c + c'$.

Using calibration with several injections at different concentrations of the standard, improved precision can be obtained. A calibration of both peak areas against standard concentration gives us two calibration curves with intercept zero or near zero and two slopes proportional to both enantiomers.

$$A = m_R + n,\ A' = m_S + n'$$

where m_R and m_S are the slopes of the linear calibration graphs and n and n' the intercepts (near zero). From this, the percentage of each enantiomer in the mixture can be calculated by (Sanchez *et al.*, 2008a):

$$\%R = [m_R/m_R + m_S] \times 100 \text{ and } \%S = [m_S/m_R + m_S] \times 100$$

The analytical signals were measured with three different detection methods: UV–vis, CD and fluorimetric. In Fig. 2 (PMT) and Fig. 3 (TMP) the chromatographic profiles obtained with the various detection methods are depicted. As can be seen, separation of enantiomers at the baseline occurs with all detectors. Some delay in the eluted peaks appearing with the fluorescence detector is a result of the tubing length in the last detector in the chromatographic system.

Operating as indicated in the chromatographic method, in reversed-phase mode with a chiral AGP column, mobile phase PBS 20 mM, pH 4.15–isopropanol (99.5:0.5, v/v) and flow-rate 0.8 mL/min, values of *Rs* (resolution) and *k'* (retention coefficient) obtained fulfilled the initial consideration, as indicated in Table 1. Calibration functions of area under peaks against commercial mixtures of standard (weighted) were as follows for PMT (c and c' in μg):

CD detector		
$A = 4025\ c + 1007$: $r = 0.9965$;	$S(-)$-PMT	55.02%
$A' = 3290\ c' + 118$: $r = 0.9995$;	$R(+)$-PMT	44.98%
UV–vis detector		
$A = 632373\ c + 9812$; $r = 0.9998$	$S(-)$-PMT	54.36%
$A' = 530914\ c' + 10120$; $r = 0.9960$	$R(+)$-PMT	45.65%
Fluorimetric detector		
$A = 936786\ c + 7556$; $r = 0.9971$	$S(-)$-PMT	54.29%
$A' = 788485\ c' + 8167$; $r = 0.9968$	$R(+)$-PMT	45.71%

Table 1. Chromatographic data and analytical parameters

		Promethazine					Trimeprazine				
Detector	Enantiomer	t_R[a] (min)	k' [b]	Rs[c]	α[d]	Percentage[e]	t_R (min)	k'	Rs	α	Percentage
	$S(-)$	14.90	5.93			55.02	8.67	3.13			50.80
Circular dichroism				1.32	1.26				1.20	1.29	
	$R(+)$	18.22	7.47			44.98	7.20	2.43			49.20
	$S(-)$	14.82	5.89			54.36	9.04	3.30			50.61
UV–vis				1.17	1.28				1.03	1.21	
	$R(+)$	18.40	7.55			45.65	7.81	2.72			49.38
	$S(-)$	15.68	4.74			54.29	9.44	2.46			50.90
Fluorescence				1.16	1.27				0.93	1.21	
	$R(+)$	19.21	6.03			45.71	8.28	2.03			49.10

[a]Retention time; [b]retention factor; [c]resolution factor; [d]enantioselectivity factor; [e]percentage in the sample.

The precision of the method depends mainly on the precision of the slopes calculation. Relative standard deviations of the slopes were calculated over four calibration curves (five points each) and the results show errors <1% were obtained in every case.

To prove the quality of the approach, we use as another case study the phenothiazine trimeprazine. Operating as indicated in the chromatographic method, in reversed-phase mode with a chiral AGP column, mobile phase PBS 20 mM, pH 3.31, and flow-rate 0.6 mL/min, the values of *Rs* (resolution) and *k'* (retention coefficient) obtained fulfilled the initial consideration, as indicated in Table 1.

The obtained results were as follows:

CD detector		
$A = 4025\ c + 137$: $r = 0.9965$;	*S*(−)-TMP	50.80%
$A' = 3290c' + 118$: $r = 0.9995$;	*R*(+)-TMP	49.20%
UV–vis detector		
$A = 151031\ c + 3590$; $r = 0.99924$	*S*(−)-TMP	50.61%
$A' = 147370\ c' + 3397$; $r = 0.9985$	*R*(+)-TMP	49.38%
Fluorimetric detector		
$A = 194490\ c + 3117$; $r = 0.9971$	*S*(−)-TMP	50.90%
$A' = 187599\ c' + 3854$; $r = 0.9990$	*R*(+)-TMP	49.10%

The obtained results in the case of TMP confirm the good performances of the methodology.

Validation procedure

The linearity of detectors response was investigated plotting calibration curves (peak area of each eluted peak against the concentration of the commercial mixture of enantiomers). The tested concentrations were prepared according with the signal sensitivity of the detectors. Linearity was assessed with four series at five concentration levels. The correlation coefficient was calculated in order to prove the linearity of the calibration curves. The obtained results are ordered in Table 2. To evaluate the intra-day precision, three control samples at different concentration levels were injected three times on the same day. The inter-day precision was determined from three independent series carried out over three consecutive days. Statistical tests were performed at a level of confidence of 95% ($p = 0.05$; Table 3).

The limits of detection and quantitation were considered to be the concentrations that produced signal-to-noise ratios of 3 and 10, respectively. They were determined by from the linear regression of both peak areas. It must be pointed out that, because no pure standards of the enantiomers are available, the concentration used in the linear regression is the total concentration of both isomers (weighted mixture). In Table 2 the results obtained regarding the analytical parameters of the method are given.

The accuracy of the methodology was estimated by plotting the obtained results with fluorimetric detection with that of UV detection, for both enantiomers. Comparison for PMT gave slopes of 1.015 ± 0.018 and 1.006 ± 0.025, intercepts of 0.0185 ± 0.037 and 0.053 ± 0.05, and correlation coefficients of 0.9995 and 0.9991, respectively. TMP gave slopes of 1.0153 ± 0.0243 and 0.9788 ± 0.0291, intercepts of -0.0312 ± 0.048, and correlation coefficients of 0.9991 and 0.9995, respectively. From these results it can be concluded that obtained values are close to ideal case ($b = 0$, $a = r = 1$).

Table 2. Analytical performances of calibration curves

Detector	Enantiomer	Promethazine						Trimeprazine					
		Range (μg)	Limit of detection (μg)	Limit of quantitation (μg)	R^2	Slope $\times 10^3$	Intercept $\times 10^3$	Range (μg)	Limit of detection (μg)	Limit of quantitation (μg)	R^2	Slope $\times 10^3$	Intercept $\times 10^3$
Circular dichroism	*S*(−)	2.0–6.0	1.00	3.30	0.9930	4.02	0.10	1.0-3.5	1.10	3.63	0.9945	0.23	0.14
	R(+)		1.10	3.63	0.9997	3.30	0.12		1.10	3.63	0.9998	0.22	0.12
UV–vis	*S*(−)	0.2–4.0	0.25	0.82	0.9996	632.37	9.81	0.2-3.5	0.31	1.02	0.9995	151.03	3.59
	R(+)		0.30	0.99	0.9725	530.91	10.12		0.03	0.09	0.9996	147.37	3.39
Fluorescence	*S*(−)	0.2–4.0	0.02	0.06	0.9942	936.78	7.55	0.2-1.5	0.02	0.06	0.9983	194.49	3.11
	R(+)		0.02	0.06	0.9936	788.48	8.16		0.02	0.06	0.9964	187.59	3.85

Table 3. Precision of obtained data

Mass injected (μg)	Promethazine						Trimeprazine					
	RSD (%) CD		RSD (%) UV–vis		RSD (%) FL		RSD (%) CD		RSD (%) UV–vis		RSD (%) FL	
	S(−)	*R*(+)	*S*(−)	*R*(+)	*S*(−)	*R*(+)	*S*(−)	*R*(+)	*S*(−)	*R*(+)	*S*(−)	*R*(+)
a												
0,2	—	—	0.59	0.53	0.79	0.81	—	—	0.57	0.56	0.78	0.83
2	0.33	0.59	0.48	0.24	0.19	0.30	0.28	0.50	0.46	0.25	0.20	0.29
3	0.11	0.53	0.21	0.22	0.27	0.18	0.10	0.54	0.20	0.23	0.25	0.19
b												
0,2	—	—	0.63	0.58	0.91	1.03	—	—	0.63	0.65	1.12	1.15
2	0.96	1.32	0.56	0.41	0.23	0.45	0.92	1.24	0.60	0.39	0.34	0.36
4	0.58	1.45	0.31	0.34	0.31	0.21	0.73	1.28	0.33	0.40	0.37	0.21

(a) Intra-day precision. (b) Inter-day precision.

Table 4. Recovery assay of promethazine over spiked human serum samples

Promethazine *RS*(±) added	Promethazine *S*(−) added (μg) calculated	Promethazine *R*(+) added (μg) calculated	Promethazine *S*(−) found (μg)	Promethazine *R*(+) found (μg)	Percentage recovery promethazine *S*(−)	Percentage recovery promethazine R (+)
0.83	0.46	0.37	0.43	0.34	93.48	91.89
2.00	1.10	0.90	1.09	0.88	99.09	97.77
3.00	1.65	1.35	1.62	1.33	98.18	98.51

Analytical recovery

For the analytical recovery study three different quantities (μg) of PMT in spiked serum were tested (Table 4). The calculated PMT (+) and PMT (−) were obtained from the previously determined correlation curves and the percentage of each enantiomer in the commercial mixture with the fluorimetric detector. Found PMT (+) and (−) were obtained after spiking the serum solution and submitting the samples to SPE following the procedure described above. The results obtained show that some losses in the extraction procedure are produced and the recovery levels are acceptable.

Conclusion

The general problem of obtain pure enantiomer standards in enantiomeric analysis was avoided, provided the sample was pure and good chromatographic separation could be performed. The results obtained when the approach was applied to enantiomeric quantitative analysis of two phenothiazine compounds, namely promethazine and trimeprazine, showed that, even in absence of pure standards, good analytical performances can be obtained.

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