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ORIGINAL

Separation of *para*- and *meta*-Imazamethabenz-methyl Enantiomers by Direct Chiral HPLC Using a Protein Chiral Selector

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Abstract A high performance liquid chromatography direct chiral method for the determination of *p*- and *m*-imazamethabenz-methyl (IMBM) enantiomers is described. The four isomers were separated using a protein chiral AGP^{TM} column. Elution order was established by circular dichroism. Factorial design was employed for mobile phase optimization using a small number of experiments. The best experimental conditions were ACN/NH_4Ac-HAc 60 mM (3/97) at a flow rate of 0.9 mL min^{-1} in isocratic mode, detection at 247 nm wavelength and 24 °C temperature. Resolution up to 1.3 was obtained for the separation of the 4 IMBM enantiomers. The proposed method is useful to determine the *p/m* IMBM ratios and their respective enantiomers in samples containing the aforementioned herbicide. The *p/m* IMBM ratio was higher in the standard than in the sample. LOD values were between 0.15 and 0.43 mg L^{-1} and recoveries were higher than 93 %.

Keywords Column liquid chromatography · IMBM enantiomers · Protein chiral selector · Formulation analysis · *p/m* Imazamethabenz-methyl ratio

Introduction

At present, about 25 % of pesticides are chiral; that is, they exist as, at least, two isomers called enantiomers which often differ in their biological properties [1]. Pesticide chirality, unlike drug chirality, has received relatively little attention, especially as regards environmental impact and ecotoxicological risks which have been evaluated mainly on the basis of their total concentration and specific chemical structures. However, several studies have shown that in some cases, one of the enantiomers exhibited at least tenfold more acute toxicity than did the other enantiomer [2]. Chiral pesticides are currently produced, applied and released into the environment as enantiomer mixtures and the knowledge about their ecotoxicological risks is mostly related to racemates; so, the information is often incomplete and non-specific. Therefore, the need for optically pure pesticides and for analysis is increasing, mainly due to each pesticide's different biological activity and toxicity [3]. Imazamethabenz-methyl (IMBM) is a chiral herbicide of the Imidazolinone family (IMIs) containing a stereogenic centre in the imidazolinone ring. This herbicide is usually found as a mixture of 5-methyl (*meta*) and 4-methyl (*para*) isomers, whose complete enantiomer resolution would result in four chromatographic peaks. IMBM is used in modern agricultural and it is applied both to foliage and through the soil, being an inhibitor of ramified chain amino acids [4, 5]. Enantioselectivity of IMI enantiomers has been recognized, *R*-enantiomers having a greater inhibiting action on acetohydroxy acid synthase than *S*-enantiomers [6, 7]. IMBM persistence in agriculture soils is high, affecting crop rotations [8, 9]. Degradation of IMIs in the environment depends on the pH of the soil; wet and alkaline environments allow a significant biological breakdown of IMIs, while dry and acid ones bind them strongly to the soil, limiting their mobility and slowing down their biological degradation. Solar photolysis is a natural way to reduce the impact of residues, *p*-IMBM being more stable than *m*-IMBM in the environment [10, 11].

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In general, direct high performance liquid chromatography (HPLC) is a powerful technique for enantiomer analysis due to mobile phase contribution to separation mechanisms and the availability of a great number of chiral stationary phases [12–15]. In particular, chiral stationary phases (CSP) based on proteins and glycoproteins, such as AGP^{TM}, which show a great variety of applications, have been most often used for drug analysis [16, 17].

As regards using direct chiral analysis on pesticides, no evidence was found of the use of AGP protein selectors except for the alkyloxyphenoxypropionic herbicides [18]. Chiral selectors have been used for IMI enantiomer separation, mainly polysaccharide derivatives, such as cellulose tris (4-methylbenzoate), cellulose tris (3,5-dimethylphenylcarbamate), amylose tris [(*S*)-a-methylbenzylcarbamate], and amylose tris (3,5-dimethylphenylcarbamate) [6, 19–21], as well as cyclodextrin derivatives, including (2,6-di-*O*-methyl b-cyclodextrin), 2,3,6-tri-b-methyl-*β*-cyclodextrin) and 2-hydroxypropyl-*γ*-cyclodextrin [7]. Using these selectors, enantiomer separation of IMIs such as, imazethapyr, imazaquin, imazamox, imazapic, imazapyr, and their methyl derivatives have been obtained. However, it was specifically mentioned that IMBM enantiomers were not able to be separated [6]. Thus, a lack of information exists regarding the simultaneous separation of *p*- and *m*-IMBM enantiomers, there being reference only to *p*- and *m*-isomer separation [1, 6, 8, 21]; in reversed HPLC, the *p*-isomer elutes first and the *p/m* ratio in an IMBM standard is around 2.

Direct chiral HPLC analysis of more than one racemic mixture, having very different polarities and interaction mechanisms with the CSPs, involves optimization of several variables; these include eluent pH, nature and concentration of the organic modifier, ionic strength and temperature. These variables affect retention and enantioselectivity of solutes on a protein-based CSP. Conformational changes of the protein CSP are also involved [6, 20, 21]; so, optimization methods by experimental design should be useful [16]. In this paper, a direct chiral HPLC method for simultaneous *p*- and *m*-IMBM enantiomer determination using a protein as chiral selector (AGP) is described. First, the optimization of the *p*- and *m*-IMBM enantiomer separation was carried out using a factorial design method and the analytical characteristics of the IMBM enantiomers were established. Elution order was determined by circular dichroism. The proposed method was applied to *p*- and *m*- enantiomer determination and their ratio in an IMBM formulation sample.

Experimental

Standards and Reagents

Imazamethabenz-methyl (99 %) was purchased from Cymit Chemica (Germany). All organic solvents were HPLC grade; methanol (MeOH) from Romil (Teknokroma, Spain), 2-propanol (2-PrOH) and acetonitrile (ACN) were purchased from Scharlau (Spain). Anhydrous acetic acid (HAc) (99 %) and ammonium acetate (NH_4Ac) were from Sigma-Aldrich (Germany). Purified water obtained from a Mill-Q-Plus system Millipore was used.

Sample

The commercial formulation Assert 30 LA from Basf Company (Germany) was analyzed. This formulation is an avenicide supplied as a viscous liquid, whose density is 1.06 g cm^{-3} and whose pH is around 5.5–7.0 [8], its DL_{50} for ingestion by a male or female rat being >2.000 mg kg^{-1}. The label specified an IMBM content of 30 % as the only active component.

Chromatography

Experiments were performed using an HPLC system, consisting of a quaternary pump Jasco PU-2089 (Japan) with a vacuum degasser, a multiple wavelength Jasco UV-2075 detector, which was controlled by Borwin software from JMBS (France), and a Rheodyne valve 7725i with a 20 µL loop as injector. A column oven Jasco CO-2067 Plus was also used.

Enantiomers were identified with a Jasco (Tokyo, Japan) liquid chromatograph equipped with the following components: a Degassy Popular DP4003; a Jasco intelligent pump model PU-1580; a Jasco L-G- 1580-04 quaternary gradient unit; a Jasco intelligent auto sampler model AS-2055 Plus with a 100 mL sample loop; a Jasco interface modulated LC-NetII/ADC; a chiral CD detector Jasco CD-2095 equipped with a Hg–Xe lamp (150 W).

A protein chiral AGP^{TM} column (5 µm, 100 × 4.0 mm) from ChromTech was used for chiral analysis; details of this column, which is based on α_1-glycoprotein acid containing 181 amino acids were the following: molecular mass, 40,000; isoelectric point, 2.7; S–S bounds, 2; carbohydrate, 45 % and acid residues, 14 [21]. For the *p*- and *m*-isomer separation, a C_{18} Tracer Excel (3 µm, 150 × 4.6 mm) column was used. A C_8 column of the same characteristics was also tested.

Procedures

Preparation of IMBM Standard Solutions

The IMBM standard stock solution was prepared at 400 mg L^{-1} concentration level by dissolving the solid in ACN; working solutions were prepared by suitable dilution in ACN.

Enantiomer Calibration Graphs

The external calibration graphs were obtained by injecting in the HPLC system 20 μL of standard solutions in the 5–25 mg L^{-1} concentration range. These solutions always contained the same ACN/buffer concentration ratio in order to keep both pH and ionic strength constant. Absorbance was measured at 247 nm. Peak area was used for quantification. Four graphs resulted from the respective *p*- and *m*- enantiomers.

Sample Preparation

The commercial formulation solution was prepared by weighing 145.2 mg of the assert sample, and dissolving and diluting it with ACN in a 100 mL volumetric flask. Subsequently, working sample solutions were prepared by transferring 5 mL of this sample stock solution into 25 mL volumetric flasks and diluting to the mark with ACN; the ACN/buffer ratio was also kept constant. A 62.5 μL volume of this working sample solution was transferred into a chromatographic vial and it was diluted up to 0.5 mL with the mobile phase. The final concentration of the sample was about 10 mg L^{-1} for IMBM.

Reversed HPLC Method

The standard solution of IMBM (20 μL) was introduced in the injector of the chromatograph HPLC system using the following conditions: mobile phase, ACN:NH_4Ac-HAc (60 mM) at pH 4.00 (15:85); flow rate, 1.0 mL min^{-1} in isocratic mode. Isomer detection was carried out at 247 nm.

Direct Chiral HPLC–UV Method

Injection of 20 μL of the analytical solution in the chromatograph was made using the following experimental conditions: ambient temperature, 24 °C; mobile phase, ACN:NH_4Ac-HAc (60 mM) at pH 4.00 (3:97); flow rate, 0.9 mL min^{-1} in isocratic mode. Enantiomer detection was carried out at 247 nm. All experimental variables were optimized by factorial design using Statgraphic Plus 5.0 statistical software.

Enantiomer Identification by HPLC-CD

Enantiomer elution order identification was determined by HPLC using a circular dichroism detector at 247 nm wavelength; a ACN/NH_4Ac/HAc buffer (60 mM, pH 4.00) (3/97) mobile phase at 0.8 mL min^{-1} min flow rate, injecting 2 μL of a 400 mg L^{-1} IMBM standard solution.

Results and Discussions

Optimization of *p*- and *m*-IMBM Isomer Separation by Reversed HPLC in the Standard

Based on the IMBM herbicide UV absorption spectrum, the wavelength selected was 247 nm. For the *p*- and *m*-IMBM isomer separation, C_8 and/or C_{18} columns, as well as mobile phases containing different percentages of MeOH, 2-PrOH or ACN modifiers were tested. Flow rates from 0.4 to 1.0 mL min^{-1} were also tried. Since IMBM pKa values are lower than 4.0, the pH was tested in the range 2.0–4.0; anhydrous acetic acid, in several percentages, and HAc/NH_4Ac buffers were also studied.

Using an isocratic mode and modifiers such as MeOH (25–35 %), 2-PrOH (6–12 %) and ACN (10–25 %) in HAc acid media (1–10 %), the C_8 column allowed the separation of *p*- and *m*-IMBM position isomers to base line, elution order being first *m*-IMBM and then *p*-IMBM; retention time decreased notably for HAc percentages higher than 3. However, the C_{18} column did not allow this separation. When the mobile medium was changed to HAc/NH_4Ac at pH 4.00, instead of HAc alone, *p*- and *m*-IMBM position isomer separation was possible using both C_8 and C_{18} columns and the above three modifiers, but the elution order was reversed. This behavior is referred to in the literature for other acid-basic compounds [22]. Efficiency, and thus Rs, was higher in ACN mobile phases but retention times were lower in MeOH. A chromatogram obtained in the analysis of the standard is shown in Fig. 1. The elution order for the isomers was established based on literature data (4, 5, 8 and 9). The Rs between *p*- and *m*- IMBM isomers was 1.7, and assuming that response factors are the same for both enantiomers of the two isomers, and using the area percentages, the *p/m*-IMBM ratio in this standard was 4.8 ± 0.3.

Separation of *p*- and *m*-IMBM Enantiomers by Direct Chiral HPLC. Optimization by Factorial Design

The AGP™ chiral column was tested for the enantiomer separation of both *p*- and *m*-isomers. Enantioselectivity and retention capability of this column can be regulated by pH, buffer concentration, the nature and concentration of the organic modifier mobile phase, as well as temperature of the column. Given that the working pH range of the column was in the range 4–7 and the IMBM pKa was 3.5, the pH of the mobile phase was fixed at 4.00 using HAc/NH_4 buffers at several concentration levels. Modifiers such as MeOH, ACN and 2-PrOH, having percentages in the range 2–10 %, and flow rates between 0.7 and 1.1 mL min^{-1} were tried. Analysis time and modifier ratios were the lowest using ACN; so, this modifier was selected.

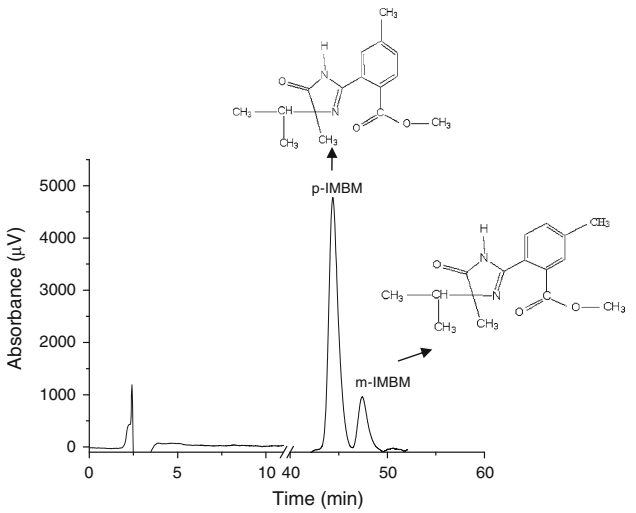


Fig. 1 Chromatograms by HPLC–UV at 247 nm from *p*/*m* IMBM isomeric separation. C_{18} column (3 mm, 100 × 4.0 mm). Mobile phase, ACN/NH_4Ac-HAc (60 mM at pH, 4.00), 15/85; flow rate 1.0 mL min^{-1}; $[IMBM]_{standard\ solution}$, 5 mg L^{-1}

Optimization by applying the one variable trial and error method was difficult because several variables were involved and mutual effects among them may occur; so, experimental design methods were tried using a small number of experiments.

Firstly, temperature (*T*), ACN percentage and NH_4Ac/HAc buffer concentration of the mobile phase were optimized by applying a 2^3 lineal factorial design (screening), which involved eight experiments. In addition, a centre point experiment, replicated two times, was carried out, giving a total of 11 experiments. These factors together with the values of their respective levels are shown in Table 1. Based on this table, the screening design was created using Statgraphic Plus 5.0 software. Responses obtained from these 11 experiments were in terms of resolution (Rs) of the *p*-IMBM enantiomer pair, peaks 2 and 3 ($Rs_{2,3}$) and peak height (*h*), of peak 2 (h_2). Factor effects on Rs and *h* indicated that positive and negative significant effects appeared. Because *T* factor has a negative significant effect on Rs and no effect on *h*, an ambient temperature of 24 °C was selected. Consequently, the main effects were ACN percentage and buffer concentration. ANOVA analysis of the resulting responses showed that these factors were statistically significant, having *p* values lower than 0.05.

This screening showed that only the buffer concentration has a positive effect on the response. The effect that the ACN percentage has on Rs is the opposite of the one it has on *h*. It should be mentioned that, in the experimental domain studied, it was difficult to evaluate resolution of the 3, 4 pair ($Rs_{3,4}$) because buffer concentration had a critical effect on peak 4 retention. This changed in a wide range, indicating that an ionic mechanism could be involved. Consequently, the above results did not allow resolution of the four enantiomers and only the separation of the first three peaks was achieved; however, the above screening analysis allowed the determination of the main effects involved, and so a more complete design was tried.

In order to determine the optimum values of the above-mentioned variables, a 3^2 factorial design of second order was applied; details of the design are specified in Table 1. Four responses were considered here; resolution between peaks 2 and 3 of *p*-IMBM enantiomers ($Rs_{2,3}$), peak 2 height (h_2), resolution between peaks 3 and 4, the latter belonging to an *m*-IMBM enantiomer ($Rs_{3,4}$),and peak 4 height (h_4). Four peaks were thus obtained; based on the *p*/*m* ratio determined above and their response factor, peaks 2 and 3 must belong to the *p*-enantiomers, which are the most abundant; tentatively, peaks 1 and 4 should belong to the *m*-enantiomers.

The general empirical model of the response surface for a second order polynomial is represented by the equation:

$$y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{l \le i \le j}^{k} b_{ij} x_i x_j \sum_{i=1}^{k} b_{ii} x_i^2$$

Table 1 Factors and settings for factorial designs

Factor	2^3 lineal factorial design		
	Level		Centre point
	+1	-1	0
C_{buffer} (mM)	70	50	60
ACN (%)	5.0	2.0	3.5
T (°C)	30	22	26
	3^2 factorial design of second order		
	Level		
	+1	-1	0
C_{buffer} (mM)	70	50	60
ACN (%)	4.5	1.5	3.0

Table 2 Regression coefficients of the response surface from the quadratic equation fitted model

Regression coefficients	Responses			
	h_2^a	h_4	$Rs_{2,3}^b$	$Rs_{3,4}$
b_0	−10,989	984	8.67	13.47
b_1	−480	211	−0.29	−1.05
b_2	357	46	−0.21	−0.26
b_{12}	7.7	1.7	0.0068	0.021
b_{11}	76	32	−0.059	−0.0126
b_{22}	−2.6	0.59	0.0017	0.00087

[a] Peak height

[b] Resolution

where "y" is the response, which is related to the factors, "x"; k is the number of factors; b_o is the intercept, and b_i, b_{ij}, b_{ii} are the regression parameters for linear, interaction and quadratic factor effects, respectively.

For this 3^2 factorial design, the equation of the fitted model was:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1x_1 + b_{22}x_2x_2$$

where $y = \text{Rs}$ or h, $x_1 = \text{ACN}$ concentration and $x_2 =$ buffer concentration. All these coefficients are shown in Table 2.

The design results obtained for h_2 were shown as Pareto charts and indicated a large positive effect from both buffer concentration and ACN percentage. Likewise, the ANOVA table indicated that these factors were statistically significant because their p values were lower than 0.05. Interaction effects were not observed.

Similarly, design results were obtained for h_4, $Rs_{2,3}$ and $Rs_{3,4}$ in terms of Pareto charts; large positive effects were observed from buffer concentration on h_2 and h_4, but high buffer concentrations had a clear negative effect on $Rs_{3,4}$. Regarding the effect of the ACN percentage, the largest positive and negative effects were on h_2 and $Rs_{2,3}$, respectively. Small interaction effects from both ACN percentage and buffer concentration on $Rs_{3,4}$ were observed.

In summary, the effects of buffer concentration and ACN percentage on response are opposing. Consequently, a compromise was required to find optimum experimental separation conditions for the four IMBM enantiomers; this can be visualized in the response surface shown in Fig. 2, which has been obtained by multiresponse analysis using the desirability function [23].

The effect of ACN percentage and buffer concentration on both Rs and k peaks of the different enantiomers is visualized in Fig. 3; the clear slope changes observed should involve a change in retention mechanism, which must be mainly ionic, explaining the retention decrease for high buffer concentrations; the largest retention decrease was observed for peak 4.

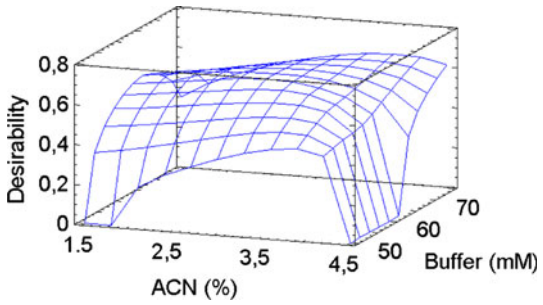


Fig. 2 Response surface of the desirability function for the 3^2 factorial design of second order

Changes in the mobile phase had strong effects on the enantiomer separation. This could be due to changes in the binding site of the protein, by charge as well as by conformational effects [24].

The chromatogram in the optimum conditions is shown in Fig. 4.

Determination of the *p*- and *m*-IMBM Ratio in the Standard Based on Enantiomers

Figure 5 shows the chromatogram obtained by injecting 20 µL of a 10 mg L^{-1} IMBM standard working solution in the optimum experimental conditions. Enantiomer elution order identification was determined by HPLC using a circular dichroism detector as shown in Fig. 5; as mentioned in "Experimental", a lesser volume of a more concentrated solution was injected to obtain sufficient resolution. The CD chromatogram shows four peaks, of which two are positive and two are negative; the (+)-enantiomer shows positive Cotton effect and the (−)-enantiomer shows negative Cotton effect. In our case, *p*-IMBM depicts two big signals (higher concentrations) situated at 10 and 13 min, which can be assigned to *p*(+)-IMBM and *p*(−)-IMBM, respectively. Likewise, *m*-IMBM shows CD signal at 8 and 15 min, corresponding to *m*(−)-IMBM and *m*(+)-IMBM. Overlapping of the 3 and 4 peaks was due to the high concentration injected; the highest k value was lesser than 12 and Rs for the critical pair was 1.3; based on the fact that response factors are the same for the two enantiomers of each isomer, percentages of *m*- and *p-isomers* were 16 ± 3 and 84 ± 6, respectively, as seen in Table 3. The *p*/*m*-isomer ratio is 5.1 ± 0.7; enantiomer ratios were close to 1. There were no significant differences at a probability level of 95 % in the *p*/*m* ratio for the standard when it was calculated by RP HPLC or by direct chiral HPLC; p value for one way ANOVA analysis was 0.052, which was slightly higher than 0.05.

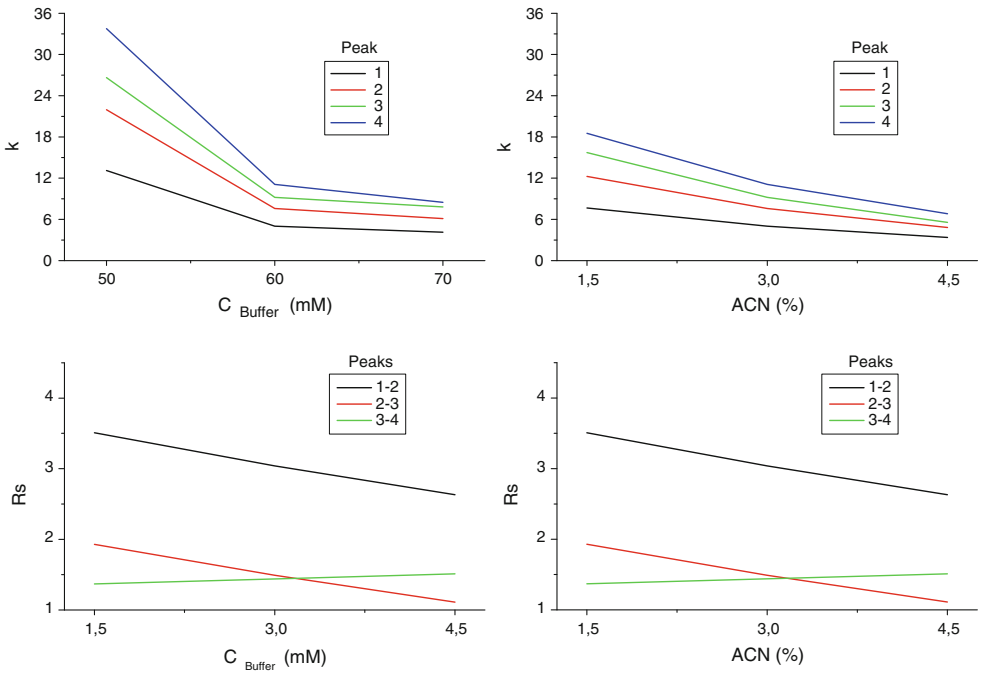
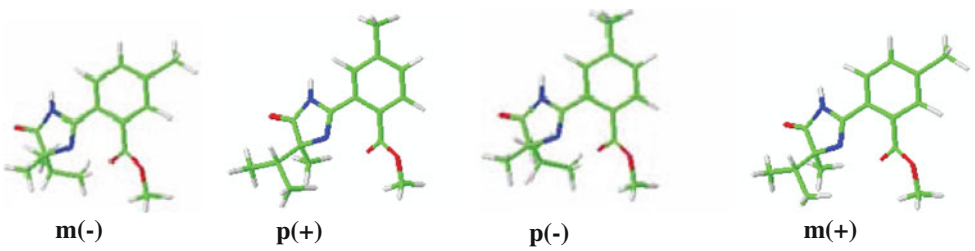


Fig. 3 Effect of different parameters on the IMBM enantiomer separation



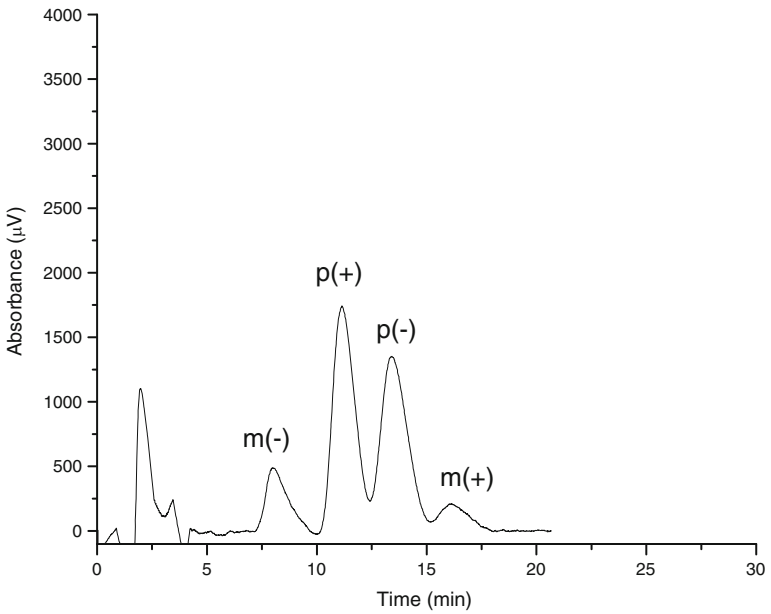


Fig. 4 Chromatograms by HPLC–UV at 247 nm from *p/m* IMBM enantiomeric separation. Protein chiral AGP™ column (5 μm, 100 × 4.0 mm). Mobile phase, ACN/NH_4Ac-HAc (60 mM at pH, 4.00), 3/97; flow rate, 0.9 mL min^{-1}; $[IMBM]_{standard\ solution}$, 5 mg L^{-1}

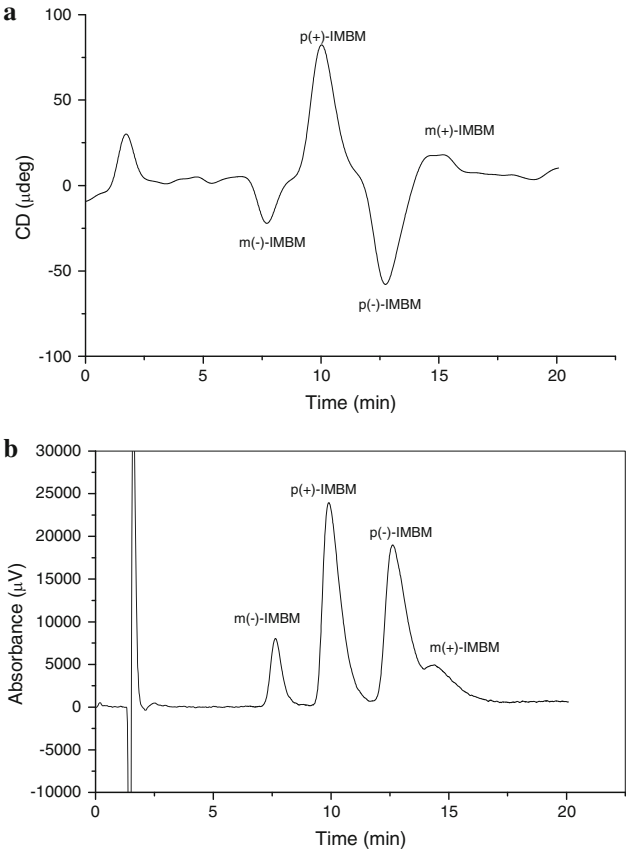


Fig. 5 Identification of the enantiomeric elution order by HPLC-CD. Chromatograms at 247 nm **a** HPLC-CD, **b** HPLC–UV. Chiral AGP™ column (5 μm, 100 × 4.0 mm). Mobile phase, ACN/NH_4Ac-HAc (60 mM at pH, 4.00), 3/97; flow rate, 0.8 mL min^{-1}; injection volume, 2 μL; [IMBM], 400 mg L^{-1}

Assuming the same molar absorptivity for both enantiomers of each *p*- and *m*-isomer, the enantiomer (+)/(−) ratio should be one in both cases; however, it is slightly higher than one. Leaving aside the possible effect of peak width increase caused by increasing retention time, this would seem to be due to the contribution of solute–stationary phase ionic interactions; experimental *m*- and *p*(+)/(−) enantiomer pair ratios, A4/A1 and A2/A3, were 0.8 and 1.1, respectively [25].

Analytical Characteristics for Standards

The external calibration graphs for the four enantiomers were obtained in the 5–25 mg L^{-1} IMBM concentration range. Linearity and other parameters are shown in Table 3.

Formulation Sample Analysis

The Assert 30 commercial formulation sample was diluted in ACN and analytical solutions were prepared by further dilution with the mobile phase. Firstly, the *p*- and *m*-IMBM isomer ratio was determined by reverse HPLC; the chromatogram obtained is shown in Fig. 6; only two peaks were obtained, from *p*-IMBM and *m*-IMBM, respectively. Their t_R and Rs were similar to those obtained from the standard. The *p*/*m* isomer ratio specified in Table 4 was lower than the one obtained from the standard.

The direct chiral HPLC method specified in "Experimental" was applied to determine the *p*- and *m*-enantiomer content of IMBM in this formulation. The resulting chromatogram is shown in Fig. 6 and the results are also summarized in Table 4. Four peaks were also obtained, each one from the respective IMBM enantiomer. Retention order was the same as the one proposed for the standard. The *p*/*m* ratio, calculated as above, is shown in Table 5; this value was also lower than that obtained from the standard; however, resolutions were similar. The *p*/*m* IMBM ratios obtained from reversed HPLC and direct chiral HPLC were compared by ANOVA; *p* value (α) from one way ANOVA analysis was 0.15 for the standard and 0.0522 for the sample, indicating that there were no significant differences at 0.05 significance level. Enantiomer ratios in both *p*- and *m*-isomers were close to 1.

Table 3 Analytical characteristics for standards by direct chiral HPLC

IMBM			(Area ± Ic[c]) (%)	RSD[d] (%)	LOD[e] (μg mL^{-1})	LOQ[f] (μg mL^{-1})
Enantiomer	Calibration equation	R^2				
m(−)	$A^a = -7{,}451.0 + 4{,}136.2 \times C^b$	0.9891	9.2 ± 0.9	4.1	0.43	2.17
p(+)	$A = -16{,}724.3 + 19{,}964.6 \times C$	0.9956	42 ± 2	1.6	0.15	0.72
p(−)	$A = -8{,}644.6 + 18{,}382.5 \times C$	0.9951	42 ± 4	4.3	0.18	0.89
m(+)	$A = 3{,}784.1 + 5{,}286.5 \times C$	0.9911	7 ± 2	13.9	0.35	1.77

[a] Enantiomer peak area

[b] Enantiomer concentration

[c] Interval of confidence

[d] Relative standard deviation at 12.5 mg L^{-1}concentration level ($n = 3$)

[e] Limit of detection, 3 N/s

[f] Limit of quantization, 10 N/s, both measuring peak height

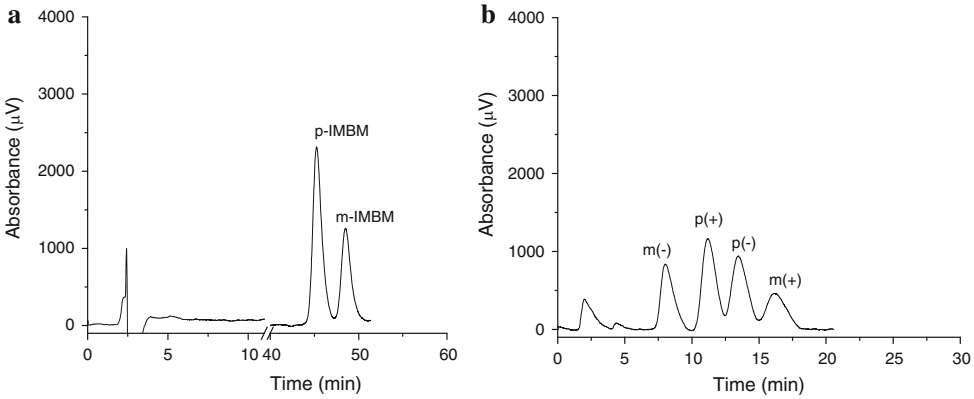


Fig. 6 Chromatograms of IMBM assert formulation by HPLC–UV at 247 nm **a** from *p/m* IMBM isomeric separation. C_{18} column (3 μm, 100 × 4.0 mm). Mobile phase, ACN/NH_4Ac-HAc (60 mM at pH, 4.00), 15/85; flow rate, 1.0 mL min^{-1}; **b** from IMBM enantiomer separation. Protein chiral AGP™ column (5 μm, 100 × 4.0 mm). Mobile phase, ACN/NH_4Ac-HAc (60 mM at pH, 4.00), 3/97; flow rate, 0.9 mL min^{-1}

Table 4 Analysis of standard and sample

HPLC method	*p/m* IMBM			
	Standard		Assert formulation sample	
	Individual data	Mean[a] ± Ic[b]	Individual data	Mean[a] ± Ic[b]
Reversed	5.02	4.8 ± 0.3	1.84	2.0 ± 0.2
	4.93		1.81	
	4.9		2.22	
	4.12		2.1	
	4.84		1.84	
Direct chiral	4.77	5.1 ± 0.7	1.7	1.76 ± 0.06
	4.79		1.69	
	5.3		1.73	
	5.2		1.81	
	5.32		1.86	

[a] *p/m* mean for $n = 5$

[b] Interval of confidence

Table 5 Analysis of the IMBM in assert sample

IMBM			RSD (%)[c] Intra-day	RSD (%)[c] Inter-day	*R* (%)[d]
Enantiomer	(Area ± Ic[a]) (%)	*C* (mg L^{-1})[b]			
m(−)	4.7 ± 0.4	4.1 ± 0.5	1.9	12.4	96.7
p(+)	11.1 ± 0.3	7.6 ± 0.3	1.5	3.2	95.0
p(−)	9.5 ± 0.7	6.5 ± 0.6	4.2	4.9	96.2
m(+)	4.5 ± 0.8	3.5 ± 0.9	7.3	20.5	93.7

[a] Confidence interval

[b] Enantiomer concentration

[c] Relative standard deviation at 12.5 mg L^{-1}concentration level ($n = 3$)

[d] Recovery at 12.5 mg L^{-1} IMBM concentration level ($n = 3$)

Conclusions

The proposed direct chiral method using an AGP column allowed simultaneous *p*- and *m*-IMBM enantiomer determination by HPLC–UV and their identification by HPLC-CD. Optimization of the method by factorial design allowed the best buffer concentration and ACN percentage to be obtained, while carrying out a small number of experiments. The *p*-isomer content was lower in the sample than in the standard. There were no significant differences in the *p/m* ratio at the 0.05 significance level calculated from the reversed HPLC and the direct chiral HPLC methods. The usefulness of the method is due to differences in the biological activity of IMBM enantiomers and their implications in agriculture and in the environment.

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