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# A validated chromatographic method for simultaneous determination of guaifenesin enantiomers and ambroxol HCl in pharmaceutical formulation

 Ola Ahmed Saleh,<sup>a</sup> Ali Mohamed Yehia,<sup>b</sup> Aida Abd-El Sattar El-Azzouny<sup>a</sup> and Hassan Youssef Aboul-Enein<sup>\*a</sup>

The performance of three phenylcarbamate based chiral stationary phases was evaluated for the optimum separation of guaifenesin enantiomers. Resolution, enantioselectivity and capacity factors were compared simultaneously using four factor three level experimental design. Chiralcel OD provided the highest resolution and selectivity but the lowest capacity factor for the less retained enantiomer along with peak broadening for the more retained enantiomer. On the other hand, Lux amylose-2 provided the lowest parameters. Optimum resolution and selectivity with the highest capacity factors was provided by Lux cellulose-2 as stationary phase and ethanol/hexane (15 : 85 v/v) as a mobile phase at a flow rate of 1.2 mL min<sup>-1</sup> and column temperature at 19 °C. Extended separation of guaifenesin enantiomers and ambroxol HCl was accomplished using the same optimized chromatographic conditions. The proposed methods were applied for the determination of analytes in syrup formulation with high specificity. The method was validated as per International Conference on Harmonization guidelines and compared with a reported HPLC method.

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## 1. Introduction

Guaifenesin (GUA); [(*R,S*)-3-(2-methoxyphenoxy)-propane-1,2-diol], is a well known expectorant which is used orally in many cases such as acute respiratory tract infections, sinusitis, bronchitis and pharyngitis. It acts by increasing the mucus flow by reducing the viscosity of bronchial secretions.<sup>1</sup> Potency and safety profile evaluation of individual enantiomers in the pharmaceutical industry is required. Although GUA is used in racemic form, a previous study assumed that one enantiomer may exhibit better physiological activity or fewer side effects.<sup>2</sup> Ambroxol HCl (AMB); *trans*-4-[(2-amino-3,5-dibromobenzyl)amino]cyclohexanol hydrochloride, is used as expectorant and mucokinetic drugs. It lowers the sputum viscosity and increases mucociliary clearance, therefore AMB decreases inflammation in airway beside its antitussive action.<sup>3,4</sup> The chemical structures of GUA enantiomers along with AMB were provided in Fig. 1.

Several analytical methods have been published in the literature for GUA and AMB determination in combination with other drugs in pharmaceutical formulations or biological fluids,

such as capillary electrophoresis,<sup>5</sup> liquid chromatography<sup>6–20</sup> and spectrophotometry.<sup>21–25</sup> On the other hand, the separation of GUA enantiomers on different chiral stationary phases (CSPs) has been adopted in the literature.<sup>26–31</sup> However, no method has been proposed for the separation and simultaneous determination of GUA enantiomers and AMB in their mixtures.

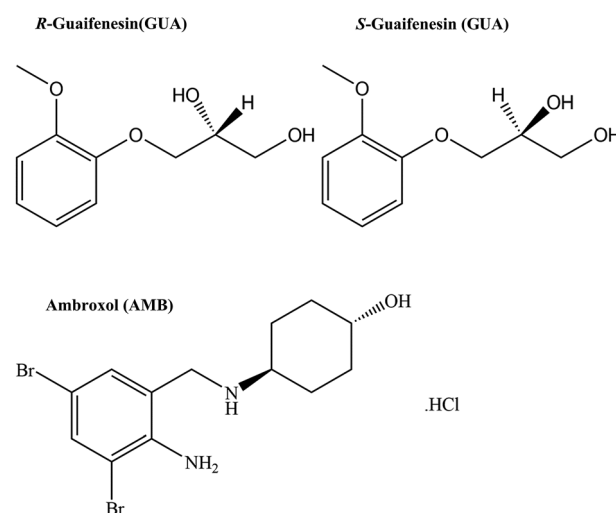


Fig. 1 Chemical structures of guaifenesin (GUA) enantiomers and ambroxol (AMB) HCl.

<sup>a</sup>Pharmaceutical and Medicinal Chemistry Department, Pharmaceutical and Drug Industries Research Division, National Research Centre (ID: 60014618), 12622 Dokki, Giza, Egypt. E-mail: haboulenein@yahoo.com; Fax: +20 33370931; Tel: +20 1003678948

<sup>b</sup>Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt

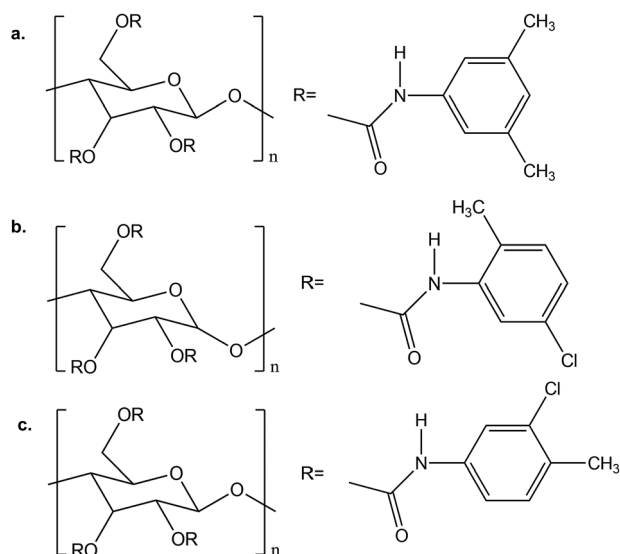


Fig. 2 Structures of chiral stationary phases (a) Chiralcel OD, (b) Lux amylose-2 and (c) Lux cellulose-2.

Based on a previous study,<sup>31</sup> the better separation of GUA enantiomers with less solvent consumption was carried out on Chiralcel OD column compared to Chiralcel AD using mixture of ethanol/hexane (30 : 70 v/v) as a mobile phase with the flow rate of 0.8 mL min<sup>-1</sup> and column temperature at 25 °C. Chiralcel OD column (CSP1) is based on the tris(3,5-dimethylphenylcarbamate) of cellulose, Fig. 2a. Recently, Lux® polysaccharide family has been introduced<sup>32,33</sup> among the commercially available CSPs. Two Lux® chiral selector stationary phases were used in this study; Lux amylose-2 (CSP2) which is based on tris(5-chloro-2-methylphenylcarbamate) of amylose, Fig. 2b and Lux cellulose-2 (CSP3); which is based on tris(3-chloro-4-methylphenylcarbamate) of cellulose, Fig. 2c. Therefore the aim of this work is to compare the efficiency of these three chiral stationary phases for the separation of guaifenesin enantiomers. An experimental design has been adopted for this purpose in order to find the chromatographic conditions for the optimum separation for GUA enantiomers. Multiple responses of resolution, enantioselectivity and capacity factors were simultaneously optimized for the studied chromatographic conditions. Furthermore, the developed method has been used for the separation of GUA enantiomers and AMB along with simultaneous determination in a pharmaceutical syrup formulation.

## 2. Experimental

### 2.1 Instrument and software

Agilent HPLC unit; 1100 series apparatus; equipped with a quaternary pump, a vacuum degasser, a column oven and a diode array UV detector. The used chiral column were Chiralcel OD column (250 × 4.6 mm i.d., 10 μm particle size) obtained from Daicel Chemical Industries, LTD (Tokyo, Japan), Lux amylose-2 and Lux cellulose-2 columns (250 × 4.6 mm, 3 μm particle size) purchased from Phenomenex (Torrance,

USA). Chromatographic data acquisition and analysis was performed by Hewlett-Packard Chemstation software for LC 3D systems; Rev. B.03.01 (317) Copyright© Agilent Technologies 2001–2007. Design of experiment was carried out using JMP® Copyright© 2012, SAS Institute Inc., Cary, NC, USA.

### 2.2 Chemicals

• Pure samples of guaifenesin (RS) and ambroxol HCl were generously obtained from Tenth of Ramadan for Pharmaceutical Industries & Diagnostic Reagents (Rameda), Sixth of October, Egypt. According to a reported HPLC method,<sup>11</sup> purity of GUA and AMB were 99.23% and 99.28%, respectively. The racemic nature of GUA pure form was confirmed by measuring its optical rotation in 95% ethanol (sodium D line, 2 dm cells)<sup>34</sup> and found to be optically inactive.

• Pharmaceutical application used was contain 15 mg of ambroxol HCl and 100 mg of guaifenesin, manufactured by Rameda, B. No. 150267.

• All solvents used in this work were of HPLC grade; methanol, ethanol and hexane (Merck, Darmstadt, Germany).

### 2.3 Standard solutions

Standard solutions of racemic GUA and AMB (1.0 mg mL<sup>-1</sup>) were prepared by accurately weighing 50 mg of their pure sample into two separate 50 mL volumetric flasks and dissolved in methanol, then volumes were made up to the mark with the same solvent.

### 2.4 Experimental design and method optimisation for GUA enantiomers separation

D-optimal design was used for the optimization of the chromatographic conditions. The design was built by JMP® custom design using one categorical factor (chiral stationary phase), one mixture factor (mobile phase) and two continuous factors (flow rate and column temperature). Nine experiments were performed evaluating four experimental factors at three factor levels, Table 1. Samples having concentration of 500 μg mL<sup>-1</sup> were prepared by suitable dilution of GUA stock solution with ethanol. The injection volume was 10 μL and UV detection was carried out at 270 nm.

Table 1 Evaluated factors and their levels for the optimization of chromatographic conditions

Experimental factor	Factor levels		
Chiral stationary phase	Chiralcel OD (CSP1)	Lux amylose-2 (CSP2)	Lux cellulose-2 (CSP3)
Mobile phase (ethanol/hexane)	10/90	20/80	30/70
Flow rate (mL min <sup>-1</sup> )	0.8	1.0	1.2
Column temperature (°C)	18	20	22

## 2.5 Method development

The optimised chromatographic conditions were applied for determination of GUA enantiomers and AMB. The separation was carried out on Lux cellulose-2 (CSP3) column thermostated at 19 °C using ethanol/hexane (15 : 85 v/v) as a mobile phase at flow rate 1.2 mL min<sup>-1</sup>. 10 µL of the samples was injected and UV detection was carried out at 270 nm. Separate standard solutions of GUA and AMB were injected in order to construct their corresponding calibration curves, while system suitability test was performed using five replicate injections of mixed standard solutions.

## 2.6 Method validation

The optimized method for the separation of GUA enantiomers and AMB was validated according to ICH guidelines.<sup>35</sup> Where, specificity, accuracy and precision were determined. Linearity ranges were confirmed along with limits of detection and quantitation. Robustness was also assisted by deliberate small variation in the proposed chromatographic conditions.

**2.6.1 Specificity.** Synthetic mixture solutions containing different ratios of GUA enantiomers and AMB were prepared from their corresponding stock solutions (1.0 mg mL<sup>-1</sup>). Analyses were performed using the optimized chromatographic conditions. System suitability parameters were checked; in addition mean recoveries and %RSD were calculated. The specificity of the developed method in presence of syrup matrix was confirmed by pharmaceutical formulation.

**2.6.2 Linearity and range.** The linearity of the method was assessed by seven concentration levels in the range of 25–250 µg mL<sup>-1</sup> for each GUA enantiomers and by six concentration levels in the range of 5–80 µg mL<sup>-1</sup> for AMB. Calibration samples were prepared by separately transferring aliquots equivalent to 0.5–5.0 mg of GUA and 0.05–0.8 mg of AMB from their respective standard solutions (1.0 mg mL<sup>-1</sup>) into 10 mL volumetric flasks and the volumes were completed with the optimised mobile phase. The calibration curves were obtained by plotting peak areas *versus* the corresponding concentrations and regression parameters were computed.

**2.6.3 Accuracy.** The accuracy of the method were evaluated by analysis of 6 pure samples for each GUA enantiomers and

AMB at concentration levels in their linear ranges and the results obtained were expressed as percent accuracy and %RSD.

**2.6.4 Precision.** Method reproducibility was determined by measuring repeatability and intermediate precision of recoveries for each GUA enantiomer and AMB. In order to determine the repeatability of the method, replicate injections ( $n = 6$ ) for each of GUA racemates (5100 and 150 µg mL<sup>-1</sup>) and AMB (10, 30 and 50 µg mL<sup>-1</sup>) were carried out. The intermediate precision was also evaluated over three days using the previously mentioned concentration levels.

**2.6.5 Limit of detection and limit of quantitation.** Limit of detection (LOD) and limit of quantitation (LOQ) were calculated using standard deviation of the response and the slope. Experimental check was done to confirm the calculated values.

**2.6.6 Robustness.** One variable at a time approach was applied in order to determine the influence of mobile phase composition, flow rate and column temperature on the proposed method. Levels of a given factor were varied while keeping the other chromatographic conditions at optimal levels previously stated in subsection 2.5. Method development. Robustness was expressed by %RSD values of peak area as qualitative response to evaluate the effect of varying ethanol percentage in mobile phase ( $\pm 1\%$ ), the flow rate ( $\pm 0.1$  units), and column temperature ( $\pm 1$  °C). Moreover, %RSD of resolution, separation and peak symmetry factors as quantitative responses were calculated, as well.

## 2.7 Assay of pharmaceutical formulation

Aliquot equivalent to 20 mg GUA and 3 mg AMB from Mucosin syrup was transferred into 100 mL volumetric flask. The volume was completed to the mark with the optimized mobile phase then filtered through a 0.40 µm membrane filter. Analysis was carried out using the previously mentioned optimized chromatographic conditions.

## 3. Result and discussion

Enantioselective chromatography has been widely applied for separation of optical isomers. When a chiral drug is in a mixture with achiral one, a chromatographic method is required to

**Table 2** The applied chromatographic conditions and their corresponding levels for the designed experiments

Experiment number	Chromatographic conditions				Retention time (min)	
	CSP	Mobile phase (ethanol/hexane)	Flow rate (mL min <sup>-1</sup> )	Column temperature (°C)	Enantiomer 1	Enantiomer 2
1	CSP1	10 : 90	0.8	18	12.97	26.18
2 <sup>a</sup>		20 : 80	1.0	20	7.14	12.76
3		30 : 70	1.2	22	4.39	5.10
4	CSP2	10 : 90	1.2	18	13.22	13.92
5 <sup>a</sup>		20 : 80	1.0	20	8.50	8.78
6		30 : 70	0.8	22	6.65	6.78
7	CSP3	10 : 90	1.2	22	16.53	17.92
8 <sup>a</sup>		20 : 80	1.0	20	9.85	10.38
9		30 : 70	0.8	18	17.76	18.31

<sup>a</sup> Experiments with centre levels.

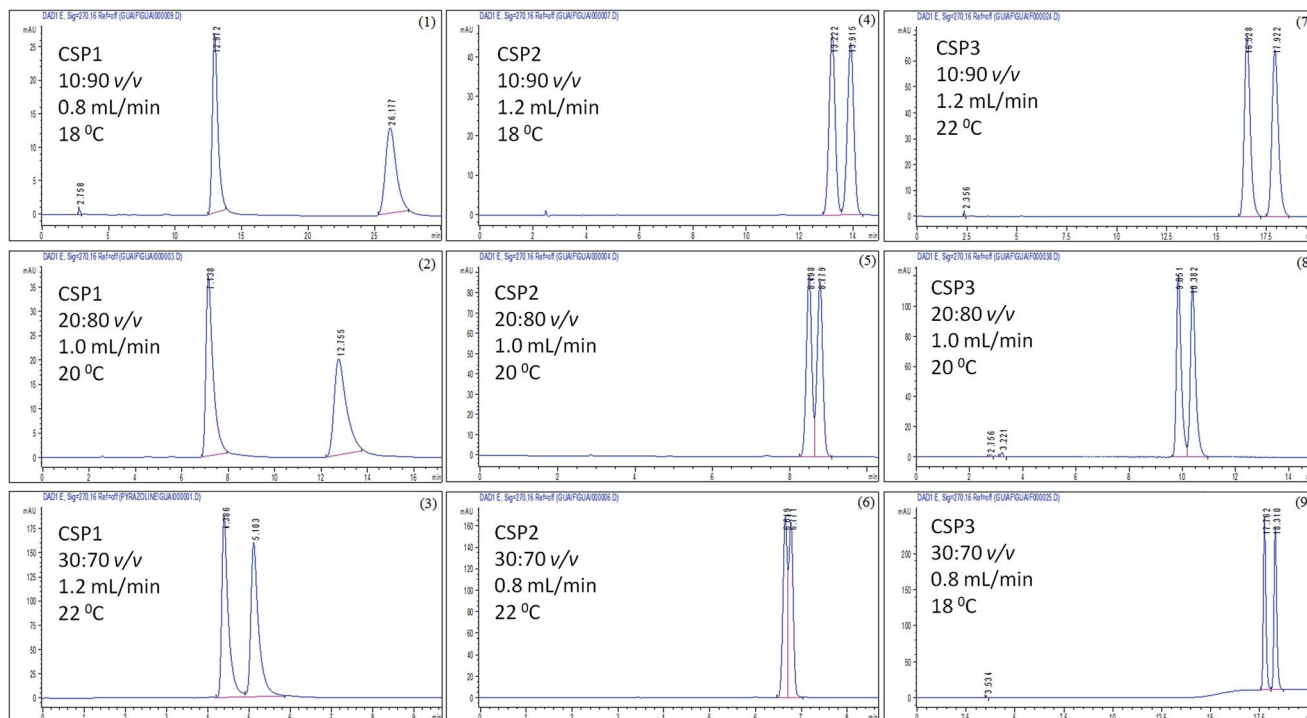


Fig. 3 Chromatograms obtained from analyses of nine experimentally designed samples for the separation of GUA enantiomer 1 (less retained) and 2 (more retained); the used chiral stationary phase, ethanol/hexane ratio in mobile phase, flow rate and column temperature are described for each chromatogram.

separate those naturally different components. A first attempt in this work was to optimize the chromatographic separation of GUA enantiomers. Therefore, experimental design was adopted

in order to identify the suitable column and optimum chromatographic conditions for enantiomeric separation in minimum number of experiments. The design also aimed to

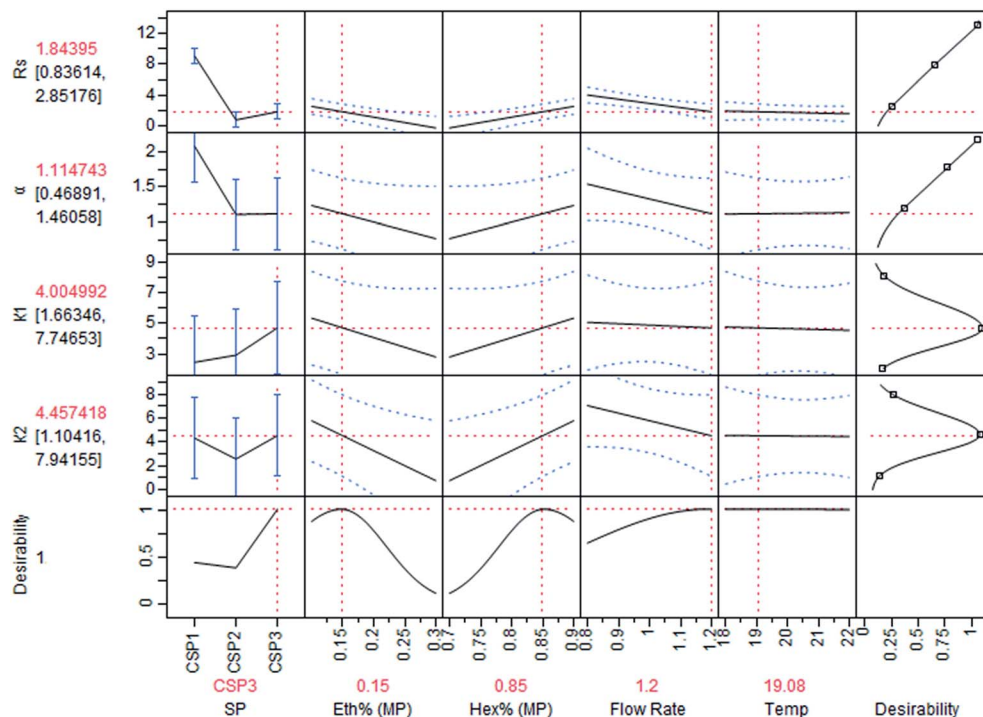


Fig. 4 Prediction profiler showing the effect of each factor on the studied responses; resolution ( $R_s$ ), selectivity factor ( $\alpha$ ), capacity factors ( $K'_1$  and  $K'_2$ ) for GUA enantiomers 1 and 2, respectively.

investigate the migration rates of GUA enantiomers on three commercial chiral stationary phases namely; Chiralcel OD (CSP1), Lux amylose-2 (CSP2) and Lux cellulose-2 (CSP3).

Nine experiments were performed using different levels for each factor and the obtained retention times of GUA enantiomers in each experiment were recorded as shown in Table 2. Generally, sufficient resolution and enantioselectivity of GUA enantiomers was observed for all the studied CSPs, Fig. 3. As the two hydroxyl groups in GUA structure, where one of them at the chiral centre, are able to form bidentate hydrogen bonds with the NH and C=O groups of the carbamate moiety present in the three CSPs.<sup>30</sup> In particular, observing the retention times of the first eluted GUA enantiomer in experiments with centre levels (samples 2, 5, and 8), Fig. 3, revealed the higher adsorption of the analyte on CSP3 (Lux cellulose-2) compared to other CSPs. This may be attributed to higher  $\pi$ - $\pi$  interaction that plays some role in the chiral discrimination.<sup>36</sup> Peak symmetry was also an important factor to consider in comparing CSPs. Apparently, GUA enantiomer 2 showed asymmetric peak with noticeable zone broadening upon using CSP1 as shown in sample 1 and 2, Fig. 3. The long residence time of GUA enantiomer 2 may increase the probability of ordinary diffusion. This effect was less pronounced in sample 3 of the same figure where analytes showed short residence time due to maximum levels of ethanol ratio in mobile phase, flow rate and column temperature used in this sample analysis.

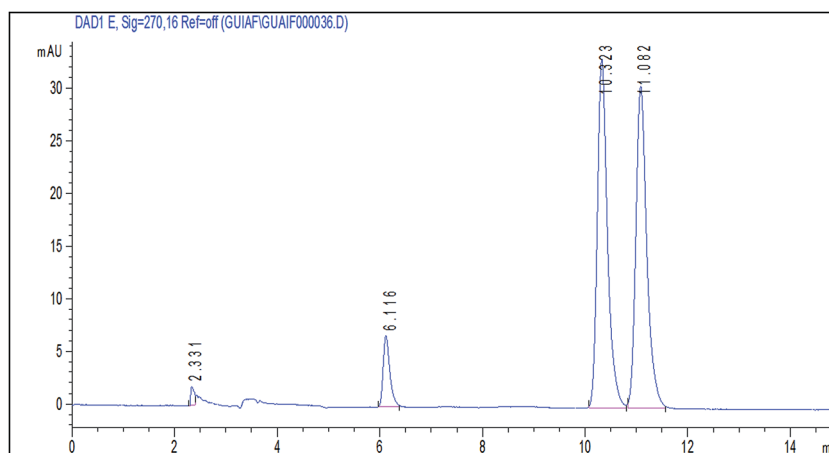
Fig. 4 showed the prediction profiler obtained by JMP® software using standard least squares fit, where the influence of each factor on resolution, enantioselectivity and capacity factors was studied. Although CSP1 (Chiralcel OD) provided the highest resolution and selectivity, it provided the lowest capacity factors for GUA enantiomer 1 ( $k'_1$ ) as shown in the profiler beside broadening in GUA enantiomer 2 observed before. Therefore, CSP3 was selected for our analysis as it provided better capacity factor along with optimum resolution. The profiler also showed the decrease in the capacity factors of enantiomers as fraction of ethanol (polar modifier) increases or hexane (non-polar

**Table 3** Regression and validation parameters for the determination of GUA enantiomers and AMB in bulk powder and results of application analysis by the proposed methods

Parameter	GUA		
	Enantiomer 1	Enantiomer 2	AMB
Selectivity (mean $\pm$ %RSD)	100.18 $\pm$ 1.93	99.98 $\pm$ 1.92	100.97 $\pm$ 1.65
<b>Linearity</b>			
Range ( $\mu\text{g mL}^{-1}$ )	25–250	25–250	5–80
Slope	4.31	6.46	2.68
Intercept	+9.67	+4.35	-1.89
Correlation coefficient ( $r$ )	0.9989	0.9991	0.9996
Accuracy (mean $\pm$ %RSD)	99.96 $\pm$ 1.34	99.39 $\pm$ 1.34	99.58 $\pm$ 1.46
<b>Precision (%RSD)</b>			
Repeatability	$\pm$ 1.97	$\pm$ 1.64	$\pm$ 1.51
Intermediate precision	$\pm$ 2.41	$\pm$ 1.99	$\pm$ 2.12
LOD ( $\mu\text{g mL}^{-1}$ )	7.93	6.38	0.35
LOQ ( $\mu\text{g mL}^{-1}$ )	24.02	19.32	1.07

modifier) decreases in the mobile phase, science residence time was decreased. In addition, flow rate and column temperature showed mild influence among the studied factors.

Prediction profiler allows simultaneous optimization on multiple responses employing a different model for each of the responses. In terms of importance, capacity factor was given the highest weight among the other responses. For maximum responses desirability, Lux cellulose-2 (CSP3) and a mobile phase consisting of ethanol/hexane (15 : 85 v/v) with a flow rate 1.2 mL min<sup>-1</sup> and column temperature at 19 °C were the optimum chromatographic conditions. These conditions provided higher capacity factors for both enantiomers along with optimum resolution and selectivity factor.



**Fig. 5** Chromatogram of pharmaceutical sample solution containing 30  $\mu\text{g mL}^{-1}$  of AMB and 100  $\mu\text{g mL}^{-1}$  of each of GUA enantiomers, separated on Lux cellulose-2 column, using ethanol/hexane (15 : 85 v/v) as mobile phase at 1.2 mL min<sup>-1</sup> flow rate and column temperature at 19 °C.

Table 4 Results of robustness study

Variable	Quantitative response (%RSD)				Qualitative response (%RSD)					
	Peak area		Resolution		Selectivity		Peak symmetry			
	GUA AMB	GUA enantiomer 1	GUA enantiomer 2	GUA enantiomer 1	GUA enantiomer 2	GUA enantiomer 1	GUA enantiomer 2	GUA AMB	GUA enantiomer 1	GUA enantiomer 2
% Ethanol	4.49	3.61	4.14	3.82	11.10	7.16	0.66	4.16	4.88	1.94
Flow rate	2.91	3.69	3.76	1.89	2.37	0.40	0.00	1.10	1.00	0.00
Column temperature	0.43	0.56	0.57	0.73	0.78	0.80	0.00	0.00	0.00	0.00

Table 5 Results of standard addition of GUA and AMB pure samples on Mucosin syrup

Mucosin syrup	Added ( $\mu\text{g mL}^{-1}$ )	Found ( $\mu\text{g mL}^{-1}$ )		Recovery <sup>a</sup> %	
		Enantiomer 1	Enantiomer 2	Enantiomer 1	Enantiomer 2
GUA (200 $\mu\text{g mL}^{-1}$ )	100	50.13	50.37	100.25	100.74
	200	101.03	101.16	101.03	101.16
	300	147.46	147.40	98.43	98.26
Mean $\pm$ %RSD				99.91 $\pm$ 1.34	100.56 $\pm$ 1.56
Mucosin syrup	Added ( $\mu\text{g mL}^{-1}$ )	Found ( $\mu\text{g mL}^{-1}$ )		Recovery <sup>a</sup> %	
AMB (30 $\mu\text{g mL}^{-1}$ )	15		15.28		101.87
	30		30.33		101.10
	45		45.74		101.65
Mean $\pm$ %RSD					101.54 $\pm$ 0.39

<sup>a</sup> Average of three determinations.

The optimized chromatographic condition succeeded not only in the separation of GUA enantiomers but also AMB separation. Fig. 5 showed three typical peaks of AMB (6.12 min), GUA enantiomer 1 (10.32 min) and enantiomer 2 (11.08 min). AMB showed lowest retention which may be attributed to its structure where the distance of cyclohexane between the OH and NH groups is unfavourable for interaction with the stationary phase, in addition to the inductive effect of electron withdrawal dibromo substitution on phenyl which probably decreases the interaction, as well.

System suitability parameters were determined and compared to reference values for sufficient separation. Resolution and selectivity factor between AMB and GUA enantiomer 1 (less retained) were 15.58 and 2.10, respectively. While resolution and selectivity factor between GUA enantiomers were 1.82 and 1.10, respectively. The capacity factors were 1.66, 3.49 and 3.82 for AMB, GUA enantiomer 1 and GUA enantiomer 2, respectively. All separated peaks were symmetrical in nature, with peak asymmetry factors around 0.8. Number of theoretical plates was around 11 000, 13 500 and

Table 6 Statistical comparison between the proposed and reported<sup>11</sup> methods for the determination of GUA and AMB in pure powder

Value	Proposed method			Reported method <sup>a</sup>	
	GUA		AMB	GUA	AMB
	Enantiomer 1	Enantiomer 2			
Mean	99.96	99.39	99.58	99.23	99.28
SD	1.34	1.33	1.45	1.804	1.336
%RSD	1.34	1.34	1.46	1.818	1.345
<i>n</i>	6	6	6	6	6
Variance	1.786	1.761	2.103	3.253	1.784
Student's <i>t</i> test (2.228) <sup>b</sup>	0.797	0.175	0.373	—	—
<i>F</i> (5.05) <sup>b</sup>	1.82	1.85	1.17	—	—

<sup>a</sup> Abdelkawy *et al.*; RPHPLC using C18 column, mobile phase; water/methanol (80 : 20 v/v) containing 1% triethylamine, pH 2.9, flow rate 1.5 mL min<sup>-1</sup> and UV detection at 220 nm. <sup>b</sup> The corresponding theoretical *t* and *F* values (*P* = 0.05).

13 400 for AMB, GUA enantiomer 1 and GUA enantiomer 2, respectively.

The method was validated according to the International Conference on Harmonization guidelines (ICH) for validation of analytical procedures.<sup>35</sup> Specificity of the method was confirmed with good mean recoveries obtained from synthetic mixtures analyses, Table 3. In addition, the separated peaks of analytes in pharmaceutical solution assay showed no interference from syrup excipients, Fig. 5. Linear regression parameters obtained from the calibration curves for each enantiomer in the specified concentration range were provided in Table 3. This table also showed good mean recoveries for pure samples assay that assisted the accuracy of the method. Repeatability and intermediate precision were checked and %RSD was calculated. The calculated LOD and LOQ were also illustrated. Robustness of the method against minor changes was examined and almost the obtained %RSD results were less than 5%, Table 4. However, higher values were obtained by changing %ethanol on the resolution and selectivity. Varying flow rate and column temperature within the specified ranges have low effect on responses as previously implied from the profiler. Besides, the column temperature showed the mildest influence on changing both quantitative and qualitative responses.

The proposed chromatographic method was applied for the analysis of syrup dosage form. Good mean recoveries  $\pm$  %RSD were obtained for AMB ( $100.42 \pm 0.99$ ) and for GUA enantiomers ( $101.14 \pm 1.26$  for enantiomer 1 and  $99.70 \pm 1.72$  for enantiomer 2). Moreover, the validity of method was assisted by applying standard addition technique, results were provided in Table 5.

Statistical comparison between the proposed and reported<sup>11</sup> HPLC methods was performed. The calculated student's *t* and *F* values were less than the tabulated values which provided that there is no significant difference regarding both accuracy and precision. However, the proposed method could perfectly separate AMB and GUA enantiomers (Table 6).

## 4. Conclusion

A comparative study on phenyl carbamates chiral stationary phases showed substantial variation in their ability to discriminate between GUA enantiomers. Chiralcel OD provided the highest resolution, however the more retained GUA enantiomer showed a broad peak. Lux amylose-2 has the lowest enantioselectivity and retention. The optimum chiral stationary phase was Lux cellulose-2 which provided high capacity factor for GUA enantiomers along with optimum resolution and selectivity. Moreover, AMB separation from GUA enantiomers was accomplished using Lux cellulose-2 and the optimized chromatographic conditions. The validated method was fast and efficient for separation of the analytes with high accuracy and precision. In addition, the proposed method showed high specificity in presence of syrup additives and therefore can be used in routine analyses.

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