Drug Development and Industrial Pharmacy, 35:19–25, 2009

ISSN: 0363-9045 print / 1520-5762 online
DOI: 10.1080/03639040802130095

informa
healthcare

A Validated HPLC Method for Separation and Determination of Promethazine Enantiomers in Pharmaceutical Formulations

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A simple, rapid, and validated method for separation and determination of promethazine enantiomers was developed. Promethazine was separated and quantitated on a Vancomycin Chirobiotic V column (250 × 4.6 mm), using a mixture of methanol, acetic acid, and triethylamine (100:0.1:0.1%, by volume) as a mobile phase at 20°C and at a flow rate of 1 mL/min. The UV-detector was set to 254 nm. Acetyl salicylic acid (Aspirin®) was used as an internal standard. The applied HPLC method allowed separation and quantification of promethazine enantiomers with good linearity (r > .999) in the studied range. The relative standard deviations (RSD) were 0.29 and 0.36 for the promethazine enantiomers with accuracy of 100.06 and 100.08. The limit of detection and limit of quantification of promethazine enantiomers were found to be 0.04 and 0.07 μg/mL, respectively. The method was validated through the parameters of linearity, accuracy, precision, and robustness. The HPLC method was applied for the quantitative determination of promethazine in pharmaceutical formulations.

Keywords chiral separation; macrocyclic glycopeptide antibiotic stationary phase; vancomycin; promethazine; pharmaceutical analysis

INTRODUCTION

Antihistaminic drugs can be broadly divided into H_1 receptor antagonists and H_2 receptor antagonists. H_1 receptors, present in smooth muscles, are readily blocked by classical antihistamines. H_2 receptors, present in stomach, stimulate gastric acid secretion (Arrang et al., 1987; Ash & Schild, 1966; Black, Duncan, Durant, Ganellin, & Parsons, 1972). H_1 receptor antagonists can be classified into six groups based on their chemical structure (Deigado & Remers, 1991; Garrison, 1992; Gay & Carliner, 1949; Martindale & Westcoot, 1993), piperazine, phenothiazine, ethylenediamine, amino alkylether, propylamine derivatives, and miscellaneous compounds. Promethazine is one of the H_1 receptor antagonists of the phenothiazine group (Figure 1). Promethazine is widely used as a potent antihistaminic for the symptomatic relief of hypersensitivity reactions or for enhancing the analgesic, anesthetic, and sedative effect of other drugs. It also has a slight hypnotic effect, which is particularly valuable in the treatment of insomnia due to allergic conditions such as asthma and pruritus. Moreover, the (+)– promethazine enantiomer has been reported to be surprisingly effective in comparison with the racemate and the (−)– enantiomer as antiosteoporotic agent. The (+)– promethazine enantiomer is effective in inhibiting the bone-resorbing cells known as osteoclasts (Boland & McDonough, 2004). In addition, the (+)– promethazine enantiomer reduces IL-6 production to 90% of the histamine-stimulated cell, whereas the (−)– enantiomer reduces to 50% in IL-6 production (McDonough, Dixon, & Nino, 2003).

The increasing use of the phenothiazine derivatives in medicine has boosted the development of several methods in their determination in pure form and in pharmaceutical preparations. Several official methods presented in British Pharmacopoeia (BP) for phenothiazines consist of nonaqueous potentiometric titrimetry or spectrophotometry in the ultraviolet region, depending on the derivative (British Pharmacopoeia, 1993). The first method is time-consuming and care must be taken with the second one because many organic compounds absorb in this region of spectrum. Various alternative methods have been reported and include titrimetry with different electrodes or in aqueous phase (Basavaiah & Krishnamurthy, 1999), spectrophotometry in the visible region, after oxidation of the phenothiazine (Basavaiah & Swamy, 2001; Karpinska, Kojlo, Grudniewska, & Puzanowska-Tarasiewicz, 1996), spectrofluorimetry (Mellinger & Keeler, 1964), differential pulse

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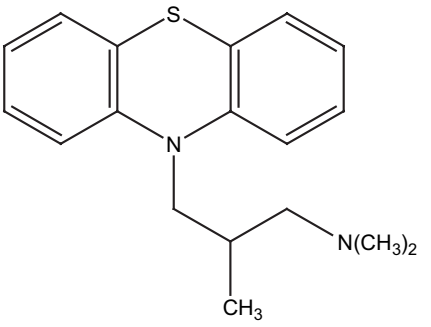

FIGURE 1. Chemical structure of promethazine.

voltammetry (DPV) (Zimova, Nemec, & Zima, 1986), differential pulse polarography (DPP) (Belal, El-Ashty, Shehata, El-Sherbeny, & El-Sherbeny, 2000), differential pulse stripping voltammetry (DPSV) (Ni, Wang, & Kokot, 2001), and electrophoresis (Wang, Lu, & Wu, 2001; Wang, Lu, Wu, & Wang, 1999). Voltammetric methods, although less selective, are easily accessible, can be useful, and turn out to be especially fast when coupled with flow injection analysis (FIA) (Calatayud, 1996; Daniel & Gutz, 2003). Also, high-performance liquid chromatography (HPLC) is used to determine stability and quantification of promethazine in human urine, serum, and postmortem materials. (Allender & Archer, 1984; Bosàkovà, Kloučkovà, & Tesarovà, 2002; Chauhan & Seshardi, 2006; De Orsi, Gagliardi, & Tonelli, 1996; Fox & McLoughlin, 1993; Leelavathi, Dressler, Soffer, Yachetti, & Knowles, 1985; Liu & Stewart, 1997; Ponder & Stewart, 1995; Tanaka et al., 2007).

Because promethazine enantiomers demonstrate different pharmacological activities, the objective of this work is to develop a validated method of enantioselective analysis of promethazine enantiomers in the pharmaceutical syrup formulation. All the validation parameters are performed including accuracy, precision, and robustness besides linearity, limit of quantification (LOQ), and limit of detection (LOD) using HPLC on a Vancomycin Chirobiotic V column. The mobile phase used was methanol:TEA:AcOH in the ratio 100:0.1:0.1 (by volume).

EXPERIMENTAL

Chemicals

Promethazine hydrochloride reference standard was obtained from Alexandria Pharmaceutical Company, Alexandria, Egypt, and Acetyl salicylic acid (Aspirin®) from Bayer Company (Leverkusen, Germany). Methanol (HPLC-grade) was obtained from Merck (Darmstadt, Germany). Acetic acid and triethylamine of analytical grades were delivered from Sigma Chemicals (St. Louis, MO, USA).

Pharmaceutical Preparation

Phenergan® Syrup was labeled to contain 5 mg promethazine hydrochloride, manufactured by Alexandria Pharmaceutical Company, Alexandria, Egypt B.No.62/8003, under license of Rhone Poulenc Rorer Paris France.

Instrumentation and Analytical Conditions

The HPLC unit was a Agilent 1100 series apparatus equipped with a quaternary pump, a vacuum degasser, a column oven, a diode array UV-detector, and a HP chemstation software. The column used was Vancomycin Chirobiotic V column of size (250 × 4.6 mm) obtained from Advanced Separation Technologies Inc. (ASTEC), Whippany, NJ, USA. The mobile phase consisted of methanol, acetic acid, and triethylamine of analytical grades (100:0.1:0.1%, by volume). The flow rate was 1.0 mL/min. All the samples were measured at wavelength of 254 nm at 20°C.

Preparation of the Standard Solutions

Promethazine hydrochloride reference standard (5 mg) was accurately weighed, transferred to 50 mL volumetric flask, and dissolved in triethylamine (0.5 mL) and methanol (20 mL), and then completed to volume with methanol (final concentration 0.1 mg/mL). The resulting solution was sonicated for 10 min and diluted to volume.

Also, an accurately weighted acetyl salicylic acid reference standard (50 mg) was transferred to 50 mL volumetric flask and dissolved in methanol (20 mL), then completed to volume with methanol (final concentration 1 mg/mL), and then sonicated for 10 min and diluted to volume. All solutions were freshly prepared.

Determination of Promethazine Enantiomers

For construction of the calibration graph, take aliquot portions (0.2–1.5 mL) of 0.1 mg/mL promethazine standard solution into a series of 25 mL measuring flasks, add 1 mL of acetyl salicylic acid as an internal standard, and complete to volume with methanol. Inject 20 µL of the solution from each flask and record the chromatograms, maintaining the flow rate at 1 mL/min and at wavelength 254 nm. Measure the ratio of peak area corresponding to concentration of each. Construct a calibration graph representing the relation between concentration and ratio of peak area. Concentration of unknown samples could be derived from the calibration graph or calculated from the following regression equation.

$$\text{Enantiomer } \mathbf{1}\text{: } Y = 0.2079x + 0.0029,\ r = 1.00$$

$$\text{Enantiomer } \mathbf{2}\text{: } Y = 0.2079x + 0.0029,\ r = 1.00$$

where Y = peak area of sample/peak area of internal standard
x = concentration of promethazine in µg/mL
r = correlation coefficient.

During the chromatographic analysis, the following parameters were measured.

t_o: was measured based on the acetyl salicylic acid peak which is used as internal standard.

k_1 and k_2: capacity factors of the first and second eluted enantiomers and were 2.37 and 2.67, respectively.

α: selectivity factor, $\alpha = k_2/k_1 = 1.12$

R_s: resolution factor was found to be 0.97, calculated according to the following equation, $R_s = 2(t_2 - t_1)/w_1 + w_2$

where w is the baseline band width obtained by drawing tangents to the inflexion points of the chromatographic peak.

Determination of Promethazine Enantiomers in Phenergan® Syrup

In a 50-mL measuring flask, accurately measure 5 mL of phenergan® syrup (1 mg/mL) claimed to contain 5 mg of promethazine. Extract with 0.5 mL triethylamine and 40 mL methanol using a magnetic stirrer and complete to volume with methanol. Determine promethazine concentration by taking 0.2–1.5 mL into 25-mL measuring flasks, and add 1 mL acetyl salicylic acid (1 mg/mL) as an internal standard. Complete to volume with methanol and proceed as previously described before.

METHOD VALIDATION

The methods were validated according to the International Conference on Harmonization guidelines for validation of analytical procedures (ICH, 1996). ANOVA was used to verify the validity of the methods.

Linearity

The calibration curve was obtained with seven concentrations of the standard solution 0.8–6 μg/mL. The solutions were prepared in triplicate. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.

Precision

The precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). Intra-day precision was evaluated by assaying the sample, at the same concentration and during the same day. Six sample solutions 0.8 μg/mL were prepared and assayed. The intermediate precision (inter-day) was studied by comparing the assays on different days (3 days).

Accuracy

The accuracy of an analytical method is determined by how close the test results obtained by that method come to the true value. It can be determined by application of the analytical procedure to an analyte of known purity (for the drug substance) or by recovery studies, where a known amount of standard is spiked in the placebo (for drug product). In this study, a number of different solutions were prepared with a known added amount of drug substance and injected in triplicate. Percent recoveries of response factor (area and concentration) were calculated as shown in Table 1, which indicates the accuracy of the proposed method.

Robustness

The robustness HPLC method was determined by analysis of samples under a variety of conditions by making small changes in the mobile phase composition, in the flow rate (0.5–1.2 mL/min), in the temperature of the column (18–25°C), and in the wavelength (240–260 nm).

Limit of Detection and Limit of Quantification

LOD is defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified, and LOQ was defined as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy.

TABLE 1
Intra-Day and Inter-Day Accuracy and Precision Data of HPLC Method for Promethazine

Theoretical Concentration (μg/mL)	Intra-Day[a]		Inter-Day[a]	
	Accuracy (%)	Precision (RSD %)	Accuracy (%)	Precision (RSD %)
Enantiomer **1**				
0.8	99.64	0.45	99.32	0.92
1.6	100.86	0.71	99.87	0.54
2.4	101.21	0.68	99.55	0.31
Enantiomer **2**				
0.8	99.79	0.31	99.12	0.24
1.6	100.62	0.63	99.62	0.31
2.4	100.92	0.42	99.41	0.77

[a]Mean of five determinations for each concentration.

RESULTS AND DISCUSSION

Currently, there is a great interest within the pharmaceutical laboratories to develop single isomer formulations and also analytical methods to determine the enantiomeric purity of drugs.

This article deals with the enantiomeric separation and quantitation of promethazine enantiomers in bulk and in pharmaceutical syrup formulations using vancomycine chiral stationary phase as a chiral selector.

The chromatographic conditions were optimized in order to provide a reliable assay performance. Mobile phase selection was based on peak parameters, runtime, ease of preparation, and cost. A typical chromatogram is shown in Figure 2 for the analysis and separation of a sample solution of promethazine enantiomers in the presence of acetyl salicylic acid as an internal standard.

The retention time was observed at 12.85 min for enantiomer **1** and at 13.96 min for enantiomer **2**, whereas at 3.81 min for the internal standard acetyl salicylic acid.

The LOD and LOQ were obtained using the slope and standard deviation of the intercept from three curves and determined by the linear regression line and were 0.2 and 0.3 µg/mL, respectively. These values were also used in an experimental assay confirming the calculation.

The calibration curves for promethazine enantiomers were constructed by plotting concentration versus the ratio of peak area and showed good linearity in the 0.8–6 µg/mL range as shown in Figures 3 and 4.

The representative linear equations were $Y = 0.2054x + 0.001$ for enantiomer **1** and $Y = 0.2079x + 0.0029$ for enantiomer **2** with high correlation coefficients $r = 1.00$ and $r = 1.00$, respectively.

Accuracy and precision of the proposed method were assessed by performing triplicate analyses of the standard solutions. Three different concentrations, diluted with the mobile phase, were prepared in the linear range of the calibration curve and analyzed to determine intra-day variability and accuracy. The inter- and intra-day precision were calculated as the RSD%. The results and the mean values were shown in Table 1, demonstrating good precision and accuracy.

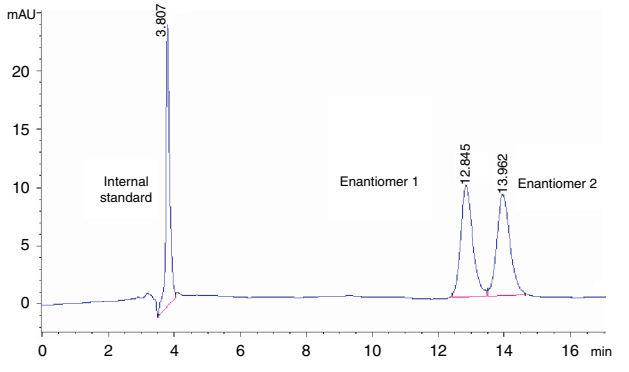

FIGURE 2. Chromatogram of promethazine 6 µg/mL on a Vancomycin Chirobiotic V column (250 × 4.6 nm) using a mixture of methanol and acetic acid and triethyl amine (100:0.1:0.1%, by volume) as a mobile phase and flow rate 1 mL/min at 254 nm.

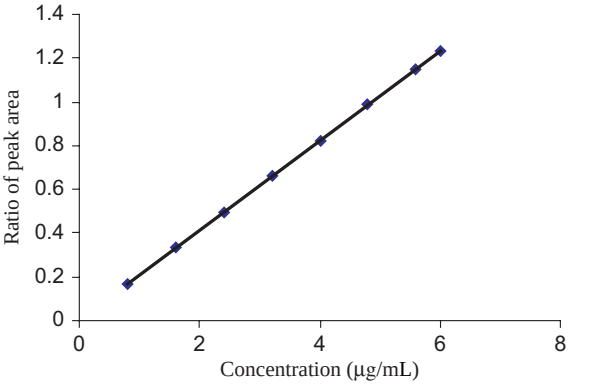

FIGURE 3. Linearity of concentration of promethazine enantiomer **1** to peak area of promethazine enantiomer **1**/peak area of internal reference standard (ratio of peak area) using the proposed HPLC procedure.

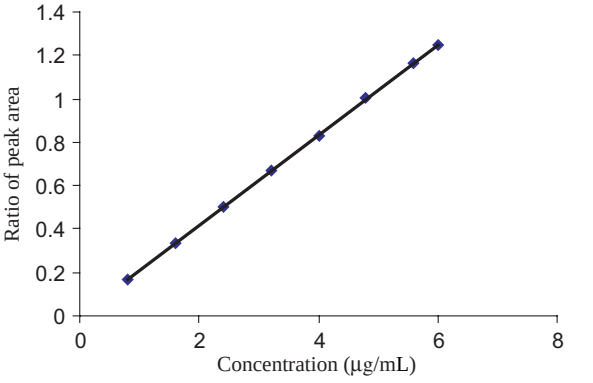

FIGURE 4. Linearity of concentration of promethazine enantiomer **2** to peak area of promethazine enantiomer **2**/peak area of internal reference standard (ratio of peak area) using the proposed HPLC procedure.

When chromatographic conditions were intentionally altered, no significant effect was observed in the chromatogram, confirming the robustness of the method.

The intra-day precision obtained by the proposed method showed a RSD of 0.61 and 0.45% for both enantiomer **1** and enantiomer **2**, respectively. Inter-day variability was calculated and showed a RSD of 0.59 and 0.44% for both enantiomers, respectively, as shown in Table 1.

Results of the determination of promethazine in Phenergan®, the pharmaceutical syrup formulation, are shown in Tables 2 and 3.

That the syrup excipients did not interfere with the analysis of promethazine enantiomers and it was found that the accuracy of the HPLC method for enantiomers **1** and **2** was 100.06 and 100.08%, respectively. The results are expressed in Table 4.

TABLE 2
Results of the Determination of Promethazine in Phenergan® Syrup by HPLC

Sample (μg)	Experimental Amount[a] (μg)	%	(RSD%)
Enantiomer **1**			
0.80	0.80	100.00	0.72
	0.81	101.25	
	0.80	100.00	
Enantiomer **2**			
0.80	0.81	101.25	1.25
	0.79	98.75	
	0.80	100.00	

[a]Mean of five determination for each concentration.

The proposed analytical method was compared with reference method (Clarke, 1986) using statistical analysis. ANOVA was applied and did not reveal a significant difference between the experimental values obtained by the two methods. The calculated *F*-value for both enantiomers (F_{cal} = 1.12) and (F_{cal} = 1.65) were found to be less than the tabulated *F*-value (F_{tab} = 6.16) and (F_{tab} = 6.16) at a 1% significance level, respectively Table 5. The methanolic solution of promethazine was stable all through the period required for analysis and did not show sign of degradation products.

Although Bosàkovà et al. (2002) reported the separation of promthazine enantiomers on vancomycin chiral stationary phase, yet the report focused on the stability study and did not discuss the validatation for quantitiation of the enantiomers in pharmaceutical formulation. The proposed method described in this article discusses a fully validated analytical procedure for promthazine enantiomers in pharmaceutical syrup formulations.

CONCLUSION

The proposed HPLC method described a quantitative determination and separation of promethazine enantiomers in bulk drug and in pharmaceutical syrup formulations. The validated HPLC method is fast, precise, accurate, and efficient and can be applied for routine analysis in quality control laboratories.

TABLE 3
Results of Standard Addition of Authentic Promethazine to Phenergan® Syrup

Added Authentic μg/mL	Found Authentic μg/mL for Enantiomer **1**	Recovery % (*x*) for Enantiomer **1**	Found Authentic μg/mL for Enantiomer **2**	Recovery % (*x*) for Enantiomer **2**
0.8	0.79	99.25	0.80	99.88
1.6	1.61	100.69	1.61	100.44
2.4	2.43	101.04	2.41	100.21
3.2	3.21	100.25	3.19	99.69
4.0	4.03	100.65	4.00	100.05
Mean ± RSD		100.38 ± 0.69		100.05 ± 0.29

TABLE 4
Determination of Authentic Promethazine via the Suggested HPLC Method

Added Authentic μg/mL	Found Authentic μg/mL of Enantiomer **1**	Recovery % of Enantiomer **1**	Found Authentic μg/mL of Enantiomer **2**	Recovery % of Enantiomer **2**
1.6	1.61	100.44	1.60	100.13
2.4	2.40	100.00	2.42	100.63
3.2	3.21	100.25	3.20	99.97
4.0	3.98	99.68	3.98	99.58
4.8	4.81	100.10	4.82	100.42
5.6	5.59	99.89	5.60	99.98
6.0	6.00	100.05	5.99	99.82
Mean ± RSD[a]		100.06 ± 0.29		100.08 ± 0.36

[a]Average of at least three separate determination.

TABLE 5
Statistical Comparison of the Results Obtained by Adopting the Proposed Method as Compared with the Reference Method[a] for Analysis of Promethazine

Technique	Mean ± RSD	*n*	Variance	Student (*t*) test	*F*
Reference method	100.02 ± 0.28	5	0.08	–	–
HPLC for Enantiomer **1**	100.06 ± 0.29	7	0.09	0.13 (2.23)[b]	1.12 (6.16)[b]
HPLC for Enantiomer **2**	100.08 ± 0.36	7	0.13	0.18 (2.23)[b]	1.65 (6.16)[b]

[a]Quantitative UV spectrophotometry in ethanol using A (1%, 1 cm) at 254 nm for the determination of promethazine.
[b]The figures in parenthesis are the theoretical *T* and *F* values at (p = .05).

ACKNOWLEDGMENTS

The authors are sincerely indebted and profoundly grateful to Professor Dr. Mohamed Nabil Aboul-Enein, Professor of Pharmaceutical Chemistry, Department of Pharmaceutical and Medicinal Chemistry, National Research Centre, for his endless support, guidance, and unlimited valuable advice throughout this work.

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