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Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 338–344
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Enantioselective determination of a gastroprokinetic drug using amylose tris-(3,5-dimethylphenylcarbamate) as a stationary phase by HPLC

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Received 26 May 2005; received in revised form 10 August 2005; accepted 11 August 2005
Available online 28 November 2005

Abstract

An enantioselective high-performance liquid chromatographic method for determination of enantiomers of mosapride citrate in bulk drugs and pharmaceuticals using UV–vis and polarimetric detectors in series has been developed. Baseline separation with resolution >2.0 was achieved on a column containing amylose tris-(3,5-dimethylphenylcarbamate) as stationary phase using a mobile phase consisting of *n*-hexane:ethanol:triethylamine (80:20:0.3, v/v/v) at 40 °C. The detection was carried out at UV-276 nm and enantiomers were identified by polarimetric detector. The effect of ethanol, 2-propanol, TEA, temperature and mobile phase flow rate on separation of MSP enantiomers was studied and the method was validated with respect to accuracy, precision, linearity and limits of detection and quantification. The linearity of the method was studied between 6.25 and 50 μg/ml and r^2 was >0.9997. The recoveries were in the range 99.63–100.22%, the method was suitable not only for process development of mosapride citrate but also for quality assurance of the individual enantiomers in bulk drugs and pharmaceuticals.

Keywords: Gastroprokinetic; Mosapride citrate; Enantioselectivity; Resolution; Polarimetric detector; Amylose tris-(3,5-dimethylphenylcarbamate)

1. Introduction

Enantiomers of a wide range of chiral drugs show significant differences in terms of their bioavailability, distribution, metabolic and excretion behaviour [1]. Very often one enantiomer represents the needed activity, while the other contributes to side effects, displays toxicity or acts as an antagonist [2,3]. With the goal of developing safer and more effective drugs, the pure active enantiomers are the ultimate products of several manufacturing units. Monitoring and determining enantiomeric purity of chiral drugs and their synthetic precursors is becoming increasingly important in pharmaceutical industry [4,5]. This has prompted development of reliable and sensitive methods capable of rendering very low quantities of an opposite enantiomer, i.e. enantiomeric purity in products. Registration of racemic drugs often demands a full documentation of pharmacological and pharmacokinetic profiles of individual enantiomers, which makes their resolution an important subject for both analytical and preparative purposes [2,6].

Mosapride citrate (MSP), known as (±)4-amino-5-chloro-2-ethoxy-*N*-[[4-(4-fluorobenz-yl)-2-morpholinyl]methyl]benzamide citrate dihydrate is a novel gastroprokinetic agent plays an important role in conjunction with life-style modifications in short and long term management of gastroesophageal reflux disease and dyspepsia in many of the Asian countries [7]. Unlike the conventional gastroprokinetic agents, it is free of dopamine D_2 receptor antagonist neither stimulates colon motor activity nor causes adverse effects such as central nervous system depression and extra pyramidal syndrome in man [8–10]. It behaves as a selective 5-HT_4-receptor agonist and enhances only the upper gastroprokinetic motor activity [11,12]. The pharmacokinetic profiles of *R*-(+) and *S*-(−) enantiomers of mosapride and its synthetic impurities remain unknown. Thus, analytical determination of the enantiomers of mosapride and its impurities is of great importance not only to evaluate their pharmacokinetics but also pharmacodynamics.

A thorough literature search has revealed that only a few analytical methods are available for determination of MSP in bulk drugs and pharmaceuticals [13–15]. Its pharmacokinetic profiles

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doi:10.1016/j.jpba.2005.08.003

in rats, dogs, monkeys and in healthy subjects [16–18] have been well characterized. Kumar et al. have studied the application of LC–MS/MS for detection of a polar impurity in the bulk drugs of MSP [19]. Recently, we have developed an achiral RP-HPLC method to determine the process-related impurities of MSP in bulk drugs and pharmaceuticals [20]. Three different methods were reported for enantioseparation of MSP using α_1-acid glycoprotein as a stationary phase. These methods include simultaneous determination of MSP and its metabolites in plasma [21]. Karlsson and Aspegren have studied the effects of mobile phase pH and column temperature on the retention and explained the reversal retention behaviour of enantiomers of MSP by calculating the entropy and enthalpy of retention process [22,23]. However, the major drawbacks of these protein-based CSPs include low capacity, lack of ruggedness and limited understanding of the mechanism of chiral recognition. Further, these are generally useful for analytical purposes but not applicable to preparative isolations. Whereas polysaccharide based stationary phases are stable and rugged when compared to protein-based materials and useful for preparative isolations. In the present investigation, we describe the enantioselective separation and determination of MSP using two different polysaccharide based CSPs, viz., cellulose tris-(3,5-dimethylphenylcarbamate) (Chiralcel OD-H) and amylose tris-(3,5-dimethylphenylcarbamate) (Chiralpak AD-H). Both the enantiomers were separated with good resolution ($R_s > 2.0$).

2. Experimental

2.1. Materials and reagents

All reagents were of analytical-reagent grade unless stated otherwise. HPLC-grade *n*-hexane (Merck, India), ethanol (Spectrochem, Mumbai, India) triethylamine (S.D. Fine Chem, Mumbai, India) were used. Samples of mosapride citrate (MSP; purity 99.95%) and process intermediates, viz., (±)2-amino-4-(4-flurobenzyl)morpholine (I), 4-amino-5-chloro-2-ethoxybenzoicacid (II) (kind of gift from Glenmark Pharmaceuticals, R&D center, Mumbai, India) were used.

2.2. Apparatus

The HPLC system composed of two LC-10AT VP pumps, an SPD-10AVP UV–vis detector, Rheodyne injector (7725i, Cotati, USA), a DGU-12A degasser and SCL-10A VP system controller (all from Shimadzu, Kyoto, Japan). Chiralcel OD-H, Chiralpak AD-H columns (25 cm × 4.6 mm i.d.; particle size 5 μm) was connected to guard column Chiralcel OD-H and Chiralpak AD-H (1 cm × 4.0 mm; 5 μm), respectively, used for separation (Daicel Chemical Industries Ltd., Tokyo, Japan). The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett-Packard, Waldron, Germany) computer system.

2.3. Chromatographic conditions

The mobile phase was *n*-hexane:ethanol:TEA (80:20:0.3, v/v/v). Before delivering into the system it was filtered through 0.45 μm, PTFE filter and degassed using vacuum. The analysis was carried out under isocratic conditions using a flow rate of 0.7 ml/min at 40 °C. Chromatograms were recorded at 276 nm using a SPD-10AVP UV–vis detector and *R*-(+), *S*-(−) enantiomers were identified by using a polarimetric detector (IBZ Messtechnik, Germany) based on their optical rotations (optical rotation range, 250; average, 10; offset, 50).

2.4. Analytical procedures

Solutions (50 μg/ml) of MSP and its intermediates were dissolved in the minimum amount of methanol and diluted with the mobile phase. The solutions were adequately diluted to study the accuracy, precision, linearity and limits of detection and quantification.

2.4.1. System suitability

The system suitability was conducted by using 50 μg/ml of two process intermediates spiked to the MSP and evaluated by making five replicate injections. The system was deemed to be suitable for use if the tailing factor for MSP and its impurities are less than or equal to 1.2 and resolution was ≥2.0. The quantities of impurities and assay of MSP enantiomers calculated from their respective peak areas. Analytical data were acquired and processed with Shimadzu Class-VP 6.12 SP_2 software.

3. Results and discussions

Cellulose and amylose-based stationary phases are generally used in a normal-phase mode with *n*-hexane containing small portions of alcohol as a modifier. Chromatographic performance (*N*), retention factor (*k*), resolution (R_s) and selectivity (α) were reported to be very affective with small changes in the composition of the mobile phase [24]. 2-Propanol and ethanol are the most commonly used modifiers which allow separation of most of the drug enantiomers on Chiralcel OD-H [cellulose tris-(3,5-dimethylphenylcarbamate)], Chiralcel OJ [cellulose tris-(3,5-dinitrophenylcarbamate] and Chiralpak AD-H [amylose tris-(3,5-dimethylphenylcarbamate)].

Fig. 1 shows the chemical reaction involved in the final step synthesis of MSP. It could be seen from Fig. 1 that intermediate I has an asymmetric carbon, which on condensation with intermediate II leads to the formation of a racemic product of MSP (1:1; *R*-(+) and *S*-(−) enantiomers) exhibiting optical isomerism. Previously, we have developed an achiral RP-HPLC method for the determination and identification of all process-related impurities of MSP. In continuation, now we have aimed at development of a chiral HPLC method for enantioselective separation and determination of MSP and its precursors not only to monitor the chemical reaction shown in Fig. 1, but also to determine the enantiomeric purity of MSP in bulk drugs and pharmaceuticals.

3.1. Column selectivity

In our preliminary experiments, cellulose (Chiralcel OD-H) and amylose (Chiralpak AD-H) stationary phases were selected.

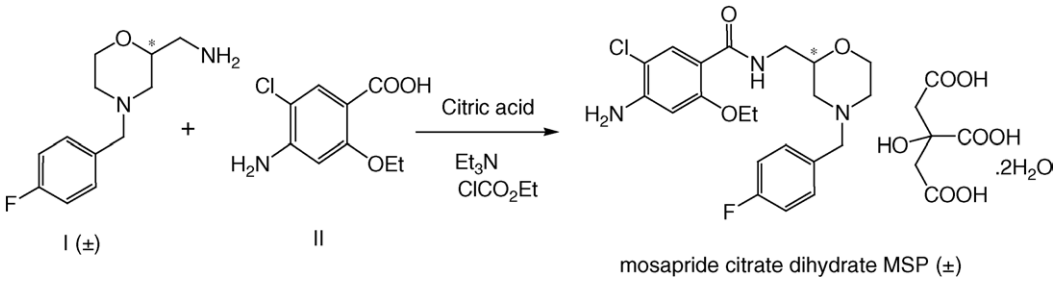

Fig. 1. Final step of the reaction involved in the synthesis of mosapride citrate dihydrate (MSP).

Polar modifiers such as ethanol and 2-propanol in different combinations with *n*-hexane were studied. The enantioselectivity and resolution data obtained are recorded in Table 1. It could be seen from the data that the enantioselectivity and resolutions were better on amylose-based stationary phase when compared to the cellulose-based stationary phase. The chiral recognition mechanism on polysaccharide based CSPs is still unclear at the molecular level. However, it has been known to some extent that the chiral resolutions were achieved through different hydrogen bonding, π–π, dipole–dipole induced interactions on these stationary phases [24–26]. The better resolution on amylose stationary phase may be due to the more number of possible interactions (viz., hydrogen bonding, π–π, dipole–dipole induced interactions) between the analytes and stationary phase.

Table 1
Resolution of MSP enantiomers on cellulose tris-(3,5-dimethylphenylcarbamate) and amylose tris-(3,5-dimethylphenylcarbamate) stationary phases using polar organic modifiers

	Cellulose stationary phase		
	k_2	R_s	α
Mobile phase			
n-Hexane:ethanol (v/v)			
95:5	2.87	0.23	1.05
90:10	3.10	0.20	1.04
80:20	6.60	0.55	1.04
80:10:10[a]	3.39	0.49	1.05
n-Hexane:2-propanol (v/v)			
90:10	13.50	0.53	1.08
80:20	7.21	0.62	1.07
70:30	2.82	0.26	1.06
60:40	1.72	0.00	1.03
Amylose stationary phase			
n-Hexane:ethanol (v/v)			
90:10	12.73	1.32	1.07
85:15	5.32	1.42	1.08
82:18	3.93	1.47	1.08
80:20	3.28	1.50	1.09
75:25	2.12	1.55	1.10
n-Hexane:2-propanol (v/v)			
90:10	16.76	0.83	1.04
80:20	6.25	0.44	1.03
75:25	3.05	0.15	1.01
70:30	1.88	0.00	1.00

Flow rate 1.0 ml/min; α separation factor; k_2 retention factor of *S*-(−)-MSP; R_s: resolution.
[a] *n*-Hexane:ethanol:2-propanol.

As amylose stationary phase has shown good enantioselectivity, it was selected for further optimization studies.

3.2. Effect of organic modifier

The effects of ethanol and 2-propanol on retention factor, enantioselectivity and resolution of MSP enantiomers were studied in the range of 10–30% (v/v) and the results are presented in Table 1. The retention times (t_R) and retention factors (k) of MSP increase with decreasing percentage of ethanol in the mobile phase, while α was not affected and decrease in R_s was observed. The same variations of retentions were observed when 2-propanol was used. These results are consistent with the decreasing ability of the solvent to displace the solute from the CSP, due to decrease of the solvent polarity [27]. When the mobile phase modifier was replaced by 2-propanol, k increased less than 40%, the variation in enantioselectivity was insignificant and the resolution slightly decreased, baseline resolution was not obtained. But in case of ethanol baseline resolution ($R_s > 1.50$) was obtained for MSP. The observed changes in retention and stereoselectivity were more likely due to the steric difference between the two molecules of solvent which may result in quite different chiral surface on the chiral stationary phase [24,28].

3.3. Effect of triethylamine (TEA)

To improve the peak shapes and the resolution, a basic additive such as triethylamine was added to the mobile phase (*n*-hexane:ethanol 80:20, v/v). TEA reduces non-specific hydrogen bonding interactions with the amylose stationary phase, reduces retention and increases efficiency by competing for these sites with the free amine on MSP. The effect of percent of TEA was studied and it could be seen from Fig. 2 that on increasing its concentration peaks became sharp and also the enantioresolution increased. At 0.3% of TEA, the highest R_s 1.58, α 1.10 was observed. As per ICH guidelines R_s value of 2.0 is preferable for quantification of enantiomers [29]. In order to improve it further, the effects of temperature and flow rate were studied. In certain cases, the flow rate appears to have quite an important influence on resolution. On increasing the flow rate, resolutions were decreased due to decreased interactions between the analyte and CSP [30]. In the present study, the effect of the flow rate on enantioresolution of MSP was studied from 1.0 to 0.5 ml/min, resolution values (R_s) were calculated according to US Pharmacopoeia (Eq. (1)) and recorded in Table 2. It could be seen from

Table 2
Effect of flow rate on resolution of MSP using *n*-hexane:ethanol:TEA (80:20:0.3, v/v/v) as mobile phase

Flow rate (ml/min)	Amylose stationary phase					
	k_1	k_2	Δt_R	$\sum W_b$	R_s	α
0.50	6.66	7.24	1.75	1.63	2.15	1.08
0.60	5.36	5.83	1.43	1.39	2.10	1.08
0.70	4.40	4.81	1.23	1.16	2.12	1.09
0.80	3.85	4.24	1.02	1.12	1.82	1.10
1.00	2.86	3.17	0.92	1.14	1.60	1.10

Flow rate variation study at 40 °C. $\Delta t_R = (t_R(S) - t_R(R))$; $\sum W_b = (W_b(S) + W_b(R))$.

Table 2, on decreasing the flow rate from 1.0 to 0.5 ml/min the resolutions were increased significantly and reached R_s 2.12 at 0.7 ml/min. This is due to the differential increase in the retention of the enantiomers ($t_R(S) - t_R(R)$) were more as compared to the peak widths at the base ($W_b(S) + W_b(R)$). These changes could be clearly seen from the data recorded in Table 2.

$$R_s = \frac{2(t_R(S) - t_R(R))}{W_b(S) + W_b(R)} \quad (1)$$

where $t_R(S)$, $t_R(R)$ and $W_b(S)$, $W_b(R)$ are retention times (in min) and base peak widths (in min) of *S* and *R* enantiomers of MSP, respectively.

3.4. Effect of temperature

The influence of column temperature on enantioselective retention was studied. The logarithm of retention factor ($\ln k$) was plotted versus inverted temperature in Kelvin. The van't Hoff plot is shown in Fig. 3. Straight lines were obtained for the both the enantiomers of MSP in the temperature range of 298–313 K. Karlsson et al., have reported the reversal of *R*-(+) and *S*-(−) enantiomers were with respect to the column temperature and pH of the mobile phase on chiral-AGP column [21–23]. This was not observed on polysaccharide based amylose stationary phase in the present investigation. The retention of both the enantiomers expressed by $\ln k$, decreased as the column temperature increased (Fig. 3). In Fig. 3, the fact that the increase in retention factors (k) with decreasing temperatures was linear with negative ΔH° reflected an enthalpically driven separation process. These results could be attributed to the fact that the analytes have small adsorption on a molecular level as temperature increases. Therefore, a faster migration through the column. This process was described by the van't Hoff equation as: $\ln k = -(\Delta H^\circ/\mathrm{RT}) + (\Delta S^\circ/R) + \ln \varphi$. This equation predicts that a plot of $\ln k$ versus $1/T$ will be a straight line with a slope of $-(\Delta H^\circ/R)$ and an intercept of $(\Delta S^\circ/R) + \ln \varphi$. The $\ln \varphi$ is independent of the temperature. In the present study, van't Hoff plots have shown a linear behaviour with the regression coefficient r^2 is 0.9897 and enthalpy ($\Delta\Delta H^\circ$), entropy ($\Delta\Delta S^\circ$) values were calculated, $\Delta\Delta H^\circ$ −0.22 k J mol^{-1}, $\Delta\Delta S^\circ$ 0.046 J K^{-1} mol^{-1}, respectively. It could be seen that the enthalpy value is more than the entropy, so that the enantioresolution of MSP enantiomers was enthalpy driven process.

Finally, the optimum conditions, viz., *n*-hexane:ethanol:TEA (80:20:0.3, v/v/v) at flow rate of 0.7 ml/min and column temperature maintained at 40 °C, were selected for the enantioselective determination of MSP. The eluents were monitored using UV–vis detector and the enantiomers were identified by polarimetric detector which was connected in series. This arrangement has made it possible to separate and detect the process key intermediates, viz., I and II of MSP and enantioresolution of I with $R_s = 2.11$ using the proposed method. The system suitability results are recorded in Table 3. Synthetic mixtures and process samples were analyzed under the identical conditions. A typical chromatogram of a synthetic mixture containing 50 μg/ml of *R*-(+) and *S*-(−) isomers spiked with 10 μg/ml each of I and II is shown in Fig. 4. Optical rotations were measured on-line using polarimetric detector to identify the *R*-(+) and *S*-(−) enan-

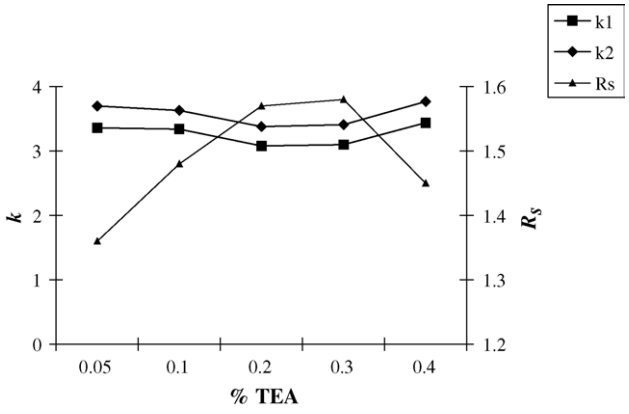

Fig. 2. Effect of TEA on retention and resolution of MSP enantiomers.

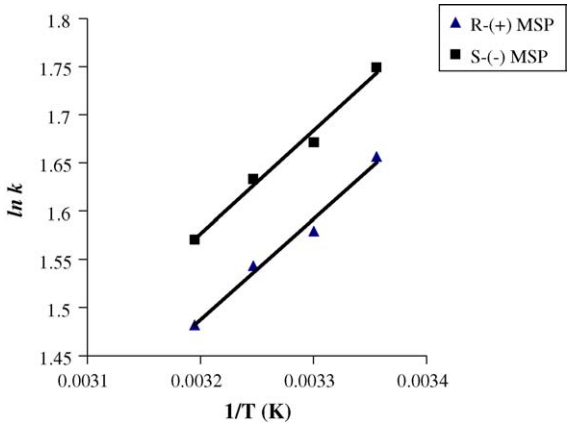

Fig. 3. Effect of column temperature on enantioselective retention of MSP enantiomers.

Table 3
Retention data

Component	t_R	k	R_s	T_f
R-(+)-MSP	16.01	4.35		1.05
S-(−)-MSP	17.50	4.78	2.12	1.20
R-(+) I	7.02	1.36		1.17
S-(−) I	7.65	1.53	2.11	1.12
II	11.98	2.83	6.70	1.15

T_f: tailing factor; t_R retention time.

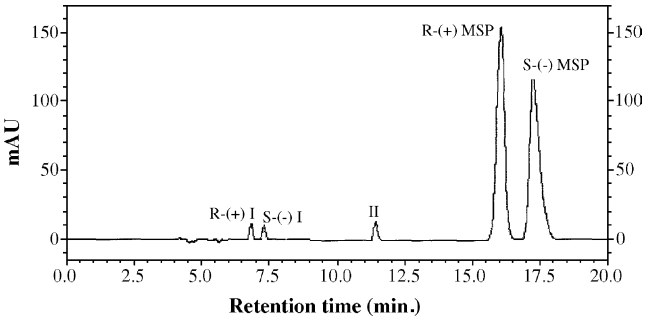

Fig. 4. Typical chromatogram of a synthetic mixture of MSP (50 μg/ml) containing process intermediates I (10 μg/ml) and II (10 μg/ml).

tiomers (Fig. 5). The method has been validated with respect to the precision, accuracy, linearity and limits of detection and quantification.

3.5. Validation

3.5.1. Precision

The precision of the method was analyzed by six ($n = 6$) injections of 25 μg/ml solution of each enantiomer and the R.S.D. (%) of retention time and peak areas was calculated. The range of R.S.D. (%) was from 0.15 to 0.50, respectively.

3.5.2. Accuracy

The recoveries of *R*-(+) and *S*-(−) enantiomers were assessed by spiking the each of the enantiomer at five different levels ranging 12.5–37.5 μg/ml. The recovery range and R.S.D. (%) for each enantiomer was 98–99.6 and 0.29–1.20%, respectively.

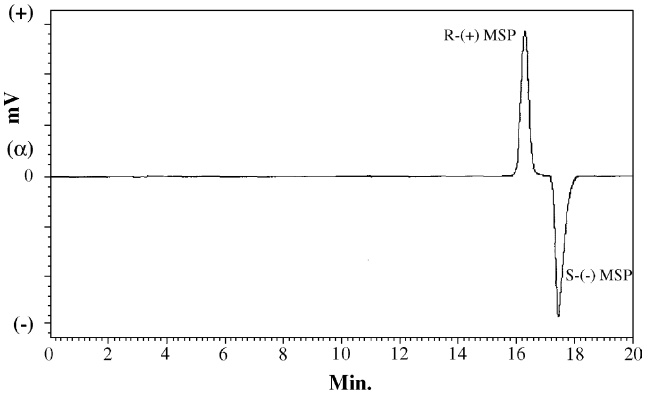

Fig. 5. Typical HPLC chromatogram of *R*-(+) and *S*-(−) enantiomers of MSP using on-line polarimetric detector.

3.5.3. Linearity

The linearity of peak area versus concentration was studied from 6.25 to 50 μg/ml for each *R*-(+) and *S*-(−) enantiomer. The data were subjected to statistical analysis using a linear-regression least-squares method. The calibration curves were found to be linear $y = 62{,}198x + 47{,}427$ and $y = 62{,}910x + 43{,}367$ with correlation coefficients (r^2) 0.9993, 0.9995 *R*-(+) and *S*-(−), respectively. The limits of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratio of 3 for LOD and 10 for LOQ, respectively. LOD and LOQ were determined by measuring the magnitude of analytical background by injecting blank samples (mobile phase) and calculating the signal-to-noise ratio for each isomer by injecting series of solutions until the S/N ratio 3 for LOD and 10 for LOQ. The results were found to be 0.12 μg/ml (LOD), 0.25 μg/ml (LOQ) for *R*-(+) and 0.6 μg/ml (LOD), 0.76 μg/ml (LOQ) for *S*-(−).

3.6. Applications

3.6.1. Process monitoring

This method was applied to monitor the progress of the final step reaction in the process development of MSP. The reaction mixtures were collected at different time intervals and the percent of enantiomers formed were determined. The samples were analyzed for 24 h and percent of each enantiomer is recorded in Table 4. These results show that the method finds application in determination of enantiomeric excess not only during the asymmetric but also racemic synthesis of MSP from intermediate I. The levels of *R*-(+) and *S*-(−) enantiomers of MSP as well as I could be monitored.

3.6.2. Analysis of bulk drugs and formulations

Further, it was used for determination of enantiomers of MSP in bulk drugs and pharmaceuticals, different tablet formulations containing mosapride citrate (equivalent to 2.5 and 5.0 mg of the active ingredient) were analyzed for determination of enantiomeric ratio by the proposed method. The two enantiomers were very well separated under the developed conditions and there was no interference from the excipients in determining the ratio of enantiomers (Fig. 6). Three batches of bulk drugs and four pharmaceutical formulations were analyzed and the impurities were not detected. The results are recorded in Table 5 and the total recoveries were found to be between 99.20 and 99.60%. From these results, it could be seen that the developed method is quite simple, rapid and reliable for determination of enantiomeric ratio and the assay of MSP in tablet formulations.

Table 4
Results of analysis of reaction mixtures collected during process development of MSP

S. no.	Reaction time (h)	%MSP	*R*-(+) (%)	*S*-(−) (%)
1	5	5.76	2.90	2.86
2	10	12.62	6.32	6.31
3	15	42.80	21.37	21.42
4	20	65.50	32.76	32.80
5	24	90.60	45.46	45.13

Table 5
Results of analysis in bulk drugs and pharmaceutical formulations

Bulk drug/formulation	Claimed value (mg)	*R*-(+)-MSP		*S*-(−)-MSP	
		Found (mg)[a]	Assay (%)	Found (mg)[a]	Assay (%)
GPL/3000/1002	52	25.792	49.60	25.844	49.70
GPL/3000/1003	50	24.85	49.70	24.90	49.80
GPL/3000/1005	50	24.87	49.75	24.92	49.85
Brand-I	2.5	1.241	49.65	1.244	49.75
Brand-II	2.5	1.243	49.75	1.246	49.85
Brand-III	5.0	2.490	49.80	2.485	49.70
Brand-IV	5.0	2.475	49.50	2.485	49.70

Claimed value is taken value in case of bulk drugs.
[a] Average of three determinations ($n = 3$).

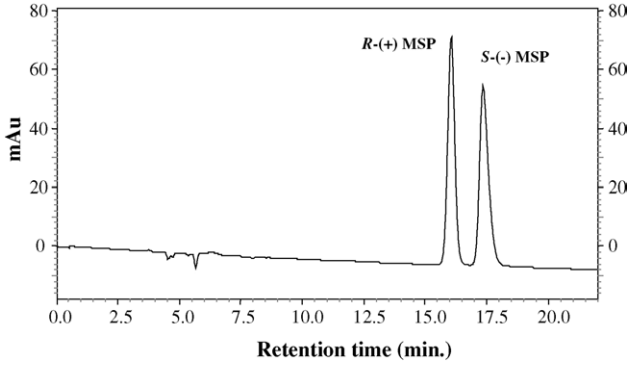

Fig. 6. Typical chromatogram of a formulation of MSP enantiomers 25 μg/ml each.

4. Conclusions

The enantioselective separation of MSP was studied on two different polysaccharide based stationary phases, viz., (i) cellulose and (ii) amylose using ethanol and 2-propanol as organic modifiers in *n*-hexane as mobile phase. The amylose-based stationary phase has yielded better resolution when compared to the cellulose. The separation was optimized to develop a simple and rapid normal-phase chiral HPLC method on polysaccharide based amylose stationary phase for separation and determination of enantiomers of MSP and its process intermediates. The method was found to be accurate and suitable for enantioselective analysis of MSP and reaction monitoring. It is also useful for determination of chiral impurities during the process development. The proposed method is rugged, robust and useful for controlling the enantiomeric purity during the asymmetric synthesis of MSP. Further, it is suitable for isolation of individual enantiomers by preparative HPLC.

Acknowledgements

The authors wish to thank Dr. J.S. Yadav, Director, Indian Institute of Chemical Technology, Hyderabad, for encouragement and permission to communicate the results for publication. Mr. D. Nagaraju thanks CSIR, New Delhi, for grant of a senior research fellowship (IICT Communication No. 050306).

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