Journal of Chromatography B, 879 (2011) 1911–1916

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Enantiomeric separation of mirtazapine and its metabolite in rat plasma by reverse polar ionic liquid chromatography using fluorescence and polarimetric detectors connected in series

R. Nageswara Rao[a,*], K. Nagesh Kumar[a], S. Ramakrishna[b]

[a] HPLC Group, Analytical Chemistry Division, Discovery Laboratory, Indian Institute of Chemical Technology, Tarnaka, Hyderabad 500607, India
[b] Pharmacology Division, Discovery Laboratory, Indian Institute of Chemical Technology, Tarnaka, Hyderabad 500607, India

ARTICLE INFO

Article history:
Received 22 January 2011
Accepted 9 May 2011
Available online 20 May 2011

Keywords:
Mirtazapine
Antidepressants
Metabolite
Vancomycin
Enantiomeric separation
Reverse polar ionic LC

ABSTRACT

A simple and rapid reverse polar ionic LC method was developed and validated for simultaneous separation and determination of mirtazapine, an antidepressant drug, and its main metabolite N-desmethyl mirtazapine using fluorescence and polarimetric detectors connected in series. The chromatographic separation was achieved on Chirobiotic V column packed with vancomycin as a stationary phase in an isocratic mode of elution of methanol:glacial acetic acid:anhydrous triethyl amine (100:0.2:0.1, v/v/v) as a mobile phase. The compounds were detected by their excitation at 290 nm and emission at 370 nm using fluorescence detector while the optical rotation (+/−) of the enantiomers was identified by polarimetric detector. The analytes were extracted from rat plasma by precipitation of proteins and the average yield was 88–111% for mirtazapine and 85–123% for N-desmethyl mirtazapine. The method was linear over the concentration range of 20–5000 ng/mL. The method was successfully applied on rat plasma spiked with the enantiomers of mirtazapine and N-desmethyl mirtazapine.

© 2011 Published by Elsevier B.V.

1. Introduction

Mirtazapine (1,2,3,4,10,14b-hexahydro-2-methyl-pyrazino [2,1-a]-pyrido [2,3-c] [2-benzazepine] (MTZ) (Fig. 1a) is a novel tetra cyclic anti depressant used not only in psychotherapy treatment but also alcoholic detoxification and post-traumatic disorders [1]. It is a stereo selective antagonist whose chirality plays an important role due to the potential of different activities and toxicities of drug enantiomers [2]. Its major metabolite, N-desmethyl mirtazapine (DMTZ) (Fig. 1b) contributes 3–6% to the total pharmacodynamic profile of the parent drug [3]. Thus it is of great importance to determine the plasma levels of the enantiomers of MTZ and DMTZ for therapeutic drug monitoring.

Several HPLC methods have been reported in the literature for analysis of MTZ and its metabolites in biological fluids. RP-HPLC [4,5] and LC–MS/MS [6] with electrospray ionization were extensively used for analysis of antidepressants including MTZ and DMTZ in plasma. Analytical and semi preparative separation of enantiomers of MTZ and its metabolites were carried out on various polysaccharide chiral stationary phases by LC [7]. Santana et al. studied the chromatographic separation of (+)-(S)- and (−)-(R)-enantiomers of MTZ on Chiralpak AD column [8]. Later it was applied to determine the enantiomers of MTZ in human plasma [9]. Mandrioli et al. used CE for enantioseparation of MTZ and DMTZ in human plasma [1]. The separation was achieved on a fused silica capillary using carboxymethyl-β-cyclodextrine dissolved in phosphate buffer at pH 2.5. However, CE lacks the sensitivity necessary to reliably determine the low levels of MTZ and its metabolite usually found in patient plasma. For this reasons a careful pretreatment using SPE with hydrophilic–lipophilic balance cartridge was proposed. Recently off-line solid-phase microextraction [10] and liquid-phase microextraction (LPME) [11] using porous polypropylene hollow fibre membrane were developed for simultaneous enantioselective determination of MTZ, DMTZ and 8-hydroxymirtazapine in plasma. However, all these procedures are not only tedious and time consuming but also involve additional steps of purification and concentration of biological samples.

Macrocyclic antibiotic CSPs have become popular for separation of a wide range of structurally different chiral compounds [12]. For example, vancomycin (Fig. 1c) produced by *Streptomyces orientalis*, has many of the separation characteristics of protein based stationary phases with exceptional stability and higher sample capacity [13]. However, so far no method has been reported in the literature using the reverse polar ionic mode of chiral separation of MTZ and DMTZ on columns packed with vancomycin as a stationary phase. The reverse polar ionic mode has many advantages not only in terms of speed and sample solubility but also beneficial for high throughput preparative separations. Mobile phases used

* Corresponding author. Tel.: +91 40 27193193; fax: +91 40 27173387.
E-mail addresses: rnrao55@yahoo.com, rnrao@iict.res.in (R.N. Rao).

1570-0232/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2011.05.017

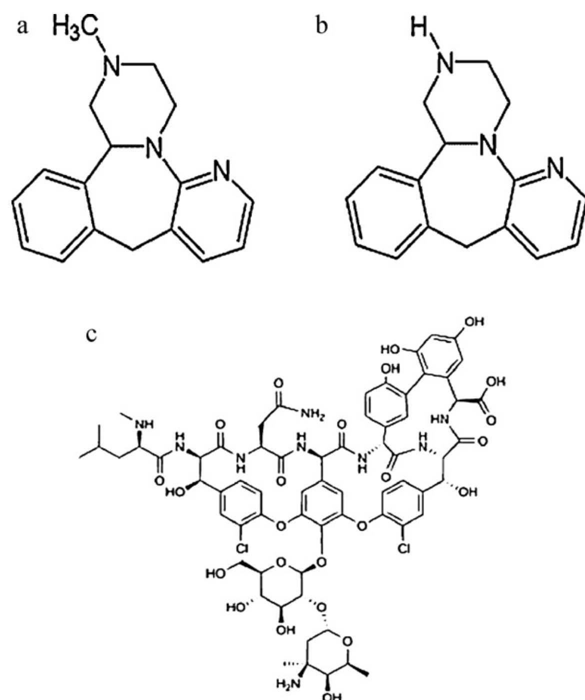

Fig. 1. Chemical structures of (a) MTZ, (b) DMTZ and (c) vancomycin.

in this mode are polar organic solvents containing volatile additives ideally suited for LC–MS applications. Thus it is of important to investigate the separation of enantiomers of MTZ and DMTZ on macrocyclic antibiotic stationary phases in reverse polar ionic mode by LC.

In the present study, the liquid chromatographic separation of enantiomers of MTZ and DMTZ was studied on a chirobiotic V column containing vancomycin as a stationary phase in reverse polar ionic mode using a mixture of methanol, acetic acid and triethyl amine as a mobile phase. The column effluents were monitored by both fluorescence and polarimetric detectors connected in series. The plasma proteins were precipitated by acetonitrile to extract MTZ and DMTZ quantitatively.

2. Experimental

2.1. Chemicals and reagents

The rac-MTZ and rac-DMTZ (purity >99.0%) obtained from Neulands laboratory (Hyderabad, India) were used. All the reagents were of analytical grade unless stated otherwise. HPLC-grade methanol, glacial acetic acid and anhydrous triethyl amine (S.D. Fine Chem; Mumbai, India) were used. Blank plasma samples were obtained from wister rats. All solvents were filtered through a 0.22 μm membrane and degassed.

2.2. Instrumentation

The HPLC system consisting of two LC-20 AD pumps, a RF-10AXL fluorescence detector, a SIL-20 AC auto sampler, a DGU-20 A_5 degasser and CBM-20A communications bus module (Shimadzu, Kyoto, Japan) was used. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packard, Waldron, Germany) computer system using LC-solution data acquiring software (Shimadzu, Kyoto, Japan). The compounds were analyzed on a Chirobiotic V column (25 cm × 4.6 mm, 5 μm) (Supelco, PA, USA). The mobile phase was filtered through a 0.22 μm membrane (Millipore) before use. A chiralyser (IBZ Messtechnik GmbH, Hannover, Germany) also known as a polarimetric detector for identification of the optical rotation (+/−) of the peaks corresponding to the enantiomers of MTZ and DMTZ was used.

2.3. Chromatographic conditions

The compounds were analyzed on a Chirobiotic V column (25 cm × 4.6 mm, 5 μm) (Supelco, PA, USA), using a mobile phase containing methanol:glacial acetic acid:anhydrous triethyl amine (100:0.2:0.1, v/v/v) in an isocratic elution mode at a flow rate of 1 mL/min. The column temperature was 20 °C. The auto sampler temperature was kept at 5 °C and the samples of 20 μL volume were injected onto the column. The data acquisition run time was 16 min. Compounds were detected by their excitation at 290 nm and emission at 370 nm using fluorescence detector while optical rotation (+/−) of MTZ and DMTZ enantiomers were identified by polarimetric detector connected in series.

2.4. Preparation of standards and quality controls

Standard 1 mg/mL stock solutions of MTZ and DMTZ were prepared separately in methanol. Standard working solutions of MTZ and DMTZ at concentrations of 0.2, 0.5, 1, 5, 10 and 50 μg/mL were prepared by serial dilution of stock solutions. Methanol was used as a diluent. Drug-free plasma was spiked with standard solutions to prepare calibration standards with final concentrations of 20, 50, 100, 500, 1000 and 5000 ng/mL of MTZ and DMTZ. Plasma quality-control (QC) samples containing MTZ and DMTZ at four concentration levels: 20 ng/mL (lower limit of quantization, LLOQ), 500 ng/mL (low, LQC), 1000 ng/mL (middle, MQC), and (5000 ng/mL) (high, HQC) were prepared to measure recovery, stability, accuracy and precision of the method. The prepared QC samples cover the therapeutic ranges of both MTZ (20–300 ng/mL) and DMTZ (50–300 ng/mL). All solutions were kept at −20 °C prior to analysis.

2.5. Plasma sample preparation

Plasma samples were stored at −80 °C and allowed to thaw gradually to room temperature before processing. After transferring 100 μL aliquots of plasma into 1.5 mL centrifuge tubes, 400 μL acetonitrile was added as a protein precipitating agent to each tube and the mixture was vortexed for 10 min and centrifuged at 4000 rpm for 20 min. Then, the upper organic layer was transferred to an autosampler vial and injected (20 μL) into the column.

2.6. Method validation

The method was validated by evaluating recovery, accuracy, precision, linearity, LOD, LOQ and stability.

2.6.1. Recovery

The recoveries were determined by comparing the peak areas of each enantiomer of MTZ and DMTZ in four different QC plasma samples (20, 500, 1000 and 5000 ng/mL) with those of each enantiomer of MTZ and DMTZ in samples prepared by spiking after deproteinization with same amounts of QC plasma samples. The recovery was calculated using the formula:

$$\text{Recovery (\%)} = \frac{\text{area of the corresponding peak in the chromatogram of rat plasma spiked with MTZ and DMTZ enantiomers and extracted}}{\text{area of the corresponding peak in the chromatogram of deproteinated blank rat plasma spiked with MTZ and DMTZ enantiomers}} \times 100$$

2.6.2. Accuracy and precision

Accuracy was evaluated by calculating the percentage deviation from the nominal concentration and reported as relative error (RE). Precision was determined by calculating the coefficient of variation (CV) of replicates within one sample run (intra-day) and between samples runs (inter-day).

For intra-day accuracy and precision, ten replicates quality-control (QC) samples containing MTZ and DMTZ, 20 ng/mL (LLOQ), 500 ng/mL (LQC), 1000 ng/mL (MQC), and (5000 ng/mL) (HQC). Samples were prepared and analyzed on the same day; and the cumulation of all five days was used for inter-day accuracy and precision determination.

2.6.3. Linearity, limit of detection (LOD) and limit of quantification (LOQ)

Linearity of the analytical method was evaluated by analyzing spiked plasma samples for each concentration ($n = 3$) over the concentration range 20–5000 ng/mL for all enantiomers of MTZ and DMTZ. The results were used to draw a linear regression curve. The LOD and LOQ were calculated according to the ICH guidelines [14].

2.6.4. Stability

The stability of analytes was determined during blood sample collection and after freezing plasma samples for 1st day, 2nd day, 3rd day, 1 week, 15 days and 1 month.

3. Results and discussion

3.1. Method development

3.1.1. Selection of chirobiotic V column in reverse polar ionic mode

The enantiomeric separation on chiral stationary phases (CSPs) is generally based on the formation of transient diastereomeric analyte–CSP complexes between the enantiomers and the chiral molecule that is an integral part of the stationary phase [15]. Vancomycin, containing 18 chiral centers with various functional groups surrounding its three pockets or cavities is an integral part of the stationary phase of chirobiotic V column [11]. The strong polar groups present on vancomycin molecule interact with the analytes groups, which are easily ionizable by the reverse polar ionic mobile

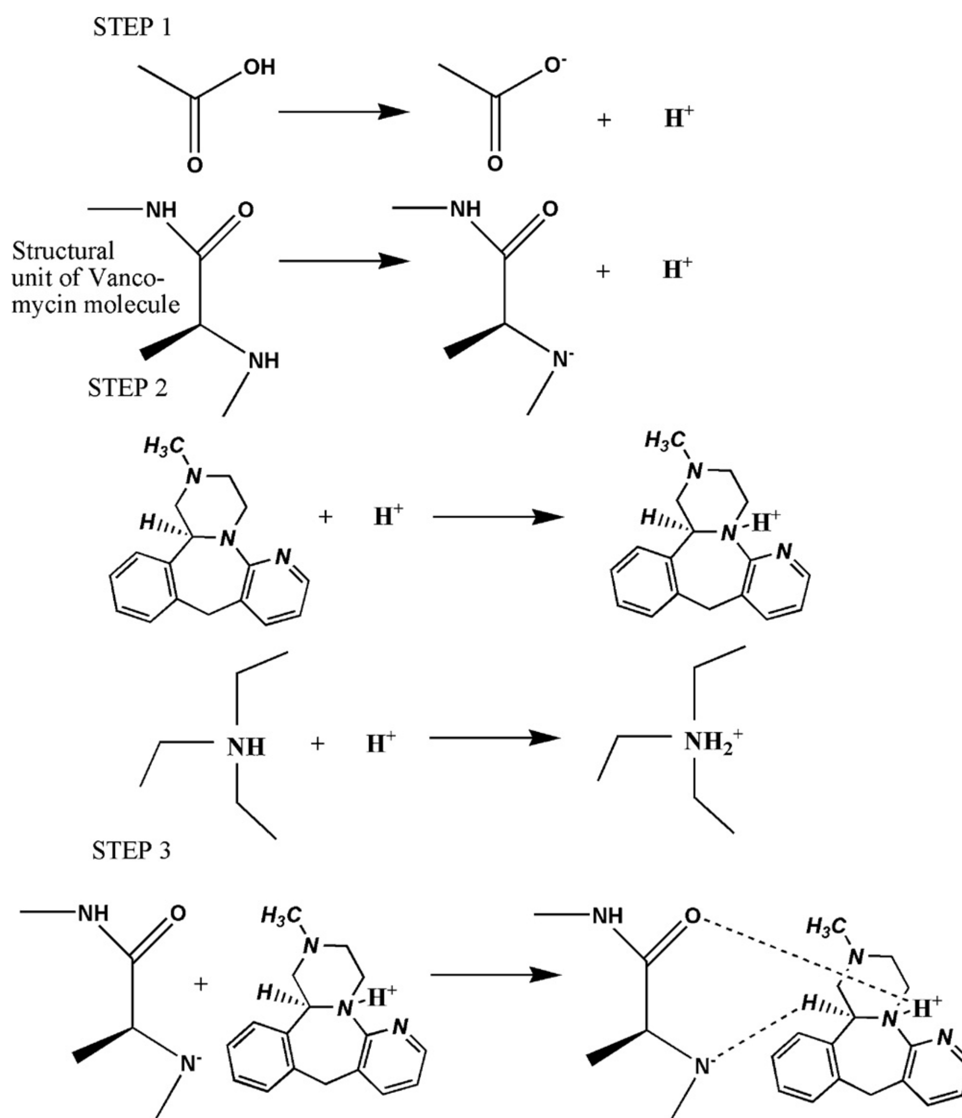

Fig. 2. Mechanism of formation of diastereomeric analyte–CSP complexes between enantiomers of MTZ/DMTZ and vancomycin. Step 1: ionization of acetic acid and vancomycin molecule. Step 2: protanation of MTZ enantiomers and triethyl amine. Step 3: interaction of ionized vancomycin molecule and protanated MTZ enantiomers, formation of transient diastereomeric analyte–CSP complexes.

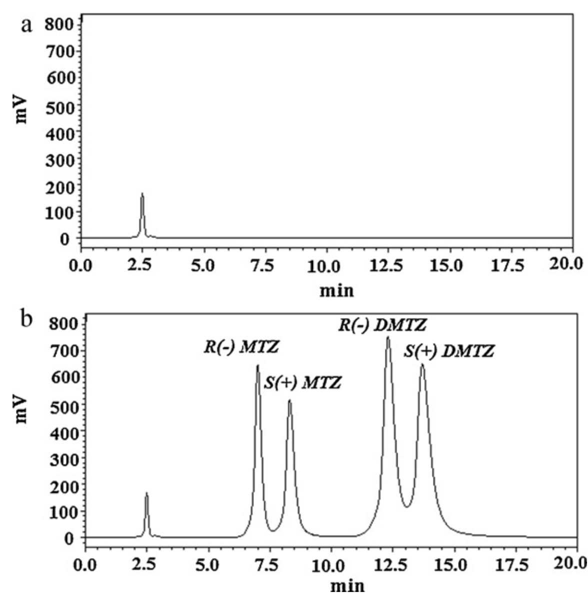

Fig. 3. Chromatograms of rat plasma (a) blank and (b) spiked with racemic MTZ and DMTZ. LC conditions: column: chirobiotic V; eluent: MeOH:AcOH:TEA (100:0.2:0.1, v/v/v); flow rate: 1 mL/min; detection: fluorescence at excitation at 290 nm and emission at 370 nm; column temperature: 20 °C.

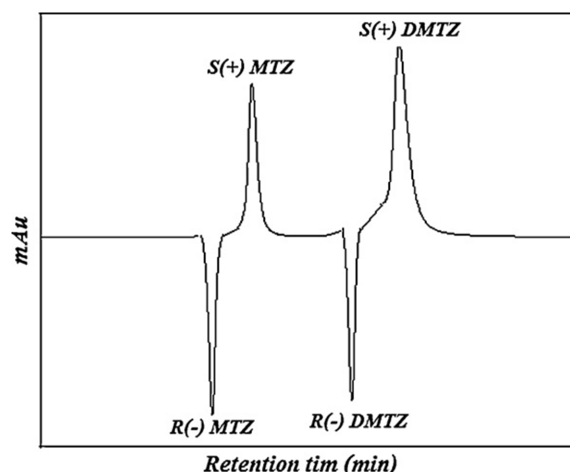

Fig. 4. A typical chromatogram showing the identification of optical rotation (+/−) of the enantiomers of standard MTZ and DMTZ by polarimetric detector. For conditions see Fig. 3.

phases. The mobile phase used in reverse polar ionic mode is useful for ionization of groups on or near the chiral centers of the analytes. The reverse polar ionic mode is applicable to all molecules with at least one ionizable group on or near the chiral center. Generally, basic compounds demonstrate more selectivity in reverse polar ionic mode [13]. MTZ and DMTZ are the basic compounds having ionizable group –N< near to chiral center. The mechanism of formation of diastereomeric analyte–CSP complexes between enantiomers and vancomycin is shown in Fig. 2. Further, the preparation of mobile phase composition is easy and the reverse polar ionic mode could be described as a novel method to obtain difficult enantioselective separation with macrocyclic antibiotic-based chiral stationary phases by LC [16].

3.1.2. Fluorescence and polarimetric detection

The compounds were detected by their excitation at 290 nm and emission at 370 nm using fluorescence detector. In the present study, baseline separation of the MTZ and DMTZ enantiomers was achieved on vancomycin CSP with fluorescence detection in the reverse polar ionic mode. The chromatographic separation of (a) blank plasma (b) rat plasma spiked MTZ, DMTZ is shown in Fig. 3. Polarimetric detector (optical rotation range, 250; average, 10 and offset is 50) connected in series was used for identification of (+/−) rotations of enantiomers of MTZ and DMTZ. The chromatographic separation of a mixture of rac MTZ and DMTZ using polarimetric detector is shown in Fig. 4. The elution order was R (−) MTZ, S(+) MTZ, R (−) DMTZ and S(+) DMTZ.

3.1.3. Mobile phase optimization

No enantiomeric separation was observed in the absence of triethyl amine when the mobile phase consisted of methanol–acetic acid (100:0.2, v/v). This could be explained on the basis of strong repulsive effects between the protonated amino groups of the analyte molecules and of the CSP. Different mobile phase compositions, flow rate, temperature of the column conditions were tried. The mobile phase composition was optimized by changing the concentration of acetic acid and keeping MeOH, TEA concentration as constant. The chiral separation was found to be very sensitive with chirobiotic V column when the concentration of AcOH was varied [13]. Initially the mobile phase composition MeOH:AcOH:TEA (100:0.1:0.1, v/v/v) was tried. The enantiomers of MTZ were eluted fast while DMTZ took long time. Later the concentration of AcOH was changed from 0.2 to 0.4%. Except at 0.2% AcOH, the enantiomeric separation of MTZ and DMTZ was not good under all the conditions. The effect of mobile phase composition on retention time (t_R), retention factor (k'), separation (α) and resolution (R_s) parameters at temperature of the column 20 °C and flow rate 1 mL/min are given in Table 1. The separation was tried at different temperatures of the column (15 °C, 20 °C, and 25 °C), and found to be good at 20 °C. Different flow rates of the mobile phase were also tried and 1 mL/min flow rate was found to be optimum. Finally, the following chromatographic conditions were optimized using the mobile phase of methanol:glacial acetic acid:anhydrous triethyl amine (100:0.2:0.1, v/v/v) in an isocratic mode of elution at a flow rate of 1 mL/min at 20 °C.

3.2. Method validation

3.2.1. Recovery

The recovery (±RSD%) of each enantiomer of MTZ and DMTZ for QC samples of 20 ng/mL (lower limit of quantization, LLOQ), 500 ng/mL (low, LQC), 1000 ng/mL (middle, MQC), and (5000 ng/mL) (high, HQC) are given in Table 2.

3.2.2. Accuracy and precision

Accuracy was evaluated by calculating the percentage deviation from the nominal concentration and is reported as relative error (RE). Precision was determined by calculating the coefficient of variation (CV) of replicates within one sample run (intra-day) and between samples runs (inter-day). Intra and inter-day accuracy and precision were determined by the performance of four concentrations of QCs and are given in Table 3.

Intra-day accuracy ranges (RE) observed for the analytes were as follows: R (−) MTZ: 0.002–0.043; S (+) MTZ: 0.005–0.025; R (−) DMTZ: 0.003–0.092; and S (+) DMTZ: 0.002–0.095. Inter-day accuracy ranges (RE) observed for the analytes were as follows: R (−) MTZ: 0.012–0.026; S (+) MTZ: 0.006–0.032; R (−) DMTZ: 0.004–0.059; and S (+) DMTZ: 0.001–0.110. Intra-day precision ranges (CV) observed for the analytes were as follows: R (−) MTZ: 0.006–0.074; S (+) MTZ: 0.013–0.056; R (−) DMTZ: 0.008–0.182; and S (+) DMTZ: 0.005–0.225. Inter-day precision ranges (CV)

Table 1
The effect of the mobile phase composition on retention and separation of enantiomers of MTZ and DMTZ.

MeOH:AcOH:TEA (v/v/v)	R (−)MTZ	S (+)MTZ	R (−)DMTZ	S (+)DMTZ
100:0.1:0.1				
t_R (min)	6.413	7.194	15.411	17.281
k'	1.338	1.623	4.619	5.301
α	–	1.213	2.846	1.148
R_s	6.297	1.019	6.649	1.091
100:0.2:0.1				
t_R (min)	6.998	8.294	12.292	13.685
k'	1.911	2.449	4.054	4.749
α	–	1.281	1.656	1.171
R_s	8.083	2.657	3.796	2.308
100:0.3:0.1				
t_R (min)	6.988	8.129	11.503	12.921
k'	1.552	1.968	3.200	3.718
α	–	1.268	1.626	1.162
R_s	6.569	1.293	2.998	0.999
100:0.4:0.1				
t_R (min)	8.632	10.536	11.879	13.542
k'	2.748	3.574	4.157	4.879
α	–	1.301	1.163	1.174
R_s	7.223	1.713	0.993	1.098

Conditions: column: Chirobiotic V; flow rate: 1 mL/min; detection: fluorescence at excitation at 290 nm and emission at 370 nm; column temperature: 20 °C.
t_R, retention time; k', capacity factor; α, selectivity factor; R_S, resolution.

Table 2
Recovery data.

Enantiomer	Recovery ± RSD (%)[a]			
	20 (ng/mL)	500 (ng/mL)	1000 (ng/mL)	5000 (ng/mL)
R (−) MTZ	99.868 ± 3.27	101.240 ± 0.24	94.995 ± 8.00	100.380 ± 2.69
S (+) MTZ	100.340 ± 7.93	92.226 ± 2.69	88.576 ± 4.66	98.601 ± 2.07
R (−) DMTZ	105.397 ± 10.43	123.022 ± 0.51	103.734 ± 19.9	85.679 ± 1.24
S (+) DMTZ	106.298 ± 17.12	119.824 ± 3.49	104.710 ± 15.71	101.245 ± 0.84

[a] $n = 3$ (triplicate determinations).

observed for the analytes were as follows: R (−) MTZ: 0.028–0.080; S (+) MTZ: 0.016–0.055; R (−) DMTZ: 0.045–0.115; and S (+) DMTZ: 0.049–0.200.

3.2.3. Linearity, limit of detection (LOD) and limit of quantification (LOQ)

Linearity of the analytical method was evaluated by analyzing spiked plasma samples for each concentration ($n = 3$) over the concentration range 20–5000 ng/mL for each enantiomer of MTZ and DMTZ. The results obtained were used to draw linear regression curve. The LOD and LOQ were calculated according to the ICH guidelines. The limit of detection (LOD), the limit of quantification (LOQ), regression equations and regression coefficients (r^2) are given in Table 4. It could be seen from Table 4 that DMTZ has higher slope (2236.9–2295.5) when compared to MTZ (1080.9–1093.6). It could be probably due to emission of more energy by DMTZ at selected excitation and emission wavelengths at 290 nm and 370 nm, respectively. Further the LOQs of both MTZ and DMTZ vary between 12.4 and 17.9 ng/mL which corresponds to the 2.48–3.58 × 10^{-10} g in an injection volume 20 μL which was almost close to the value of 3.12 × 10^{-10} g (LOQ is 6.25 ng/mL) with an injection volume 50 μL as reported by de Sanatana et al. [8,9]. This could be explained in terms of the high UV absorption of MTZ and DMTZ in normal phase solvents viz; n-hexane/iso propanol with DEA buffer where as it was found to be comparatively less in reverse polar ionic buffers viz; AcOH and TEA, which quench the UV absorption of MTZ and DMTZ significantly. Thus the fluorescence detector was used to increase the detection levels of MTZ and DMTZ in the present investigation.

Table 3
Intra- and inter-day batch accuracy and precision data.

Precision and accuracy	R (−) MTZ	S (+) MTZ	R (−) DMTZ	S (+) DMTZ
Intra-assay precision; coefficient of variation ($n = 10$)				
20 ng/mL	0.041	0.035	0.102	0.225
500 ng/mL	0.006	0.013	0.012	0.005
1000 ng/mL	0.074	0.056	0.182	0.134
5000 ng/mL	0.025	0.029	0.008	0.012
Inter-assay precision; coefficient of variation ($n = 5$)				
20 ng/mL	0.080	0.055	0.115	0.200
500 ng/mL	0.028	0.016	0.060	0.057
1000 ng/mL	0.043	0.027	0.045	0.057
5000 ng/mL	0.053	0.077	0.058	0.049
Intra-assay accuracy; relative error ($n = 10$)				
20 ng/mL	0.028	0.025	0.058	0.095
500 ng/mL	0.023	0.019	0.054	0.090
1000 ng/mL	0.002	0.005	0.003	0.002
5000 ng/mL	0.043	0.025	0.092	0.054
Inter-assay accuracy; relative error ($n = 5$)				
20 ng/mL	0.026	0.032	0.059	0.110
500 ng/mL	0.019	0.009	0.004	0.001
1000 ng/mL	0.049	0.006	0.012	0.026
5000 ng/mL	0.012	0.008	0.018	0.023

3.2.4. Stability

The stability of analytes was determined for QC concentrations except 20 ng/mL during blood sample collection and after freezing plasma samples for 1st day, 2nd day, 3rd day, 1 week, 15 days and 1 month. The results were compared with those obtained by freshly prepared samples. The results are summarized in Table 5.

Table 4
Linearity (±RSD%)[a] of slope, intercept and correlation coefficient; LOD and LOQ data.

Enantiomer	Range (ng/mL)	Regression equation	r^2	(±RSD%)[a]			LOD (ng/mL)	LOQ (ng/mL)
				m	c	r^2		
R (−) MTZ	20–5000	$y = 1093.6x - 26281$	0.9998	2.58	61.94	–	4.7	14.1
S (+) MTZ	20–5000	$y = 1080.9x - 39598$	0.9995	2.95	38.00	0.06	5.9	17.9
R (−) DMTZ	20–5000	$y = 2295.5x - 44694$	0.9988	0.76	145.89	0.11	4.1	12.4
S (+) MTZ	20–5000	$y = 2236.9x - 36318$	0.9999	1.24	152.98	0.04	4.8	14.5

"m" is slope; "c" is intercept; and"r^2" is correlation coefficient.
[a] $n = 3$ (triplicate determinations).

Table 5
Intra-and inter-stability data (±RSD%)[a] of spiked (−) MTZ.

Storage conditions	(±RSD%)[a] Nominal concentration (ng/mL)		
	500	1000	5000
Freeze/thaw stability (three cycles)	0.629	7.361	2.495
3 days	1.320	5.576	2.875
1 week	1.184	18.240	0.859
15 days	0.498	13.409	1.223
1 month	0.859	0.498	1.223

[a] $n = 3$ (triplicate determinations).

4. Conclusions

Enantiomeric separation of MTZ and its metabolite DMTZ was achieved on a new chirobiotic V column packed with vancomycin in a reverse polar ionic phase by LC. The method was developed and validated for determination of MTZ and DMTZ enantiomers in rat plasma. The repeatability of the HPLC-fluorescence-polarimetric detector was acceptable. The method showed adequate sensitivity, linearity, precision and accuracy.

Acknowledgements

The authors thank Dr. J.S. Yadav, Director, IICT and Dr. M. Vairamani, Head, Analytical Chemistry Division, IICT for encouragement and permission to communicate the results for publication. Mr. K.N. Kumar thanks Council of Scientific and Industrial Research (CSIR), New Delhi, India for a research fellowship. Also the authors thank M/s Neulands laboratories, Hyderabad for providing samples of MTZ and DMTZ.

References

[1] R. Mandrioli, V. Pucci, C. Sabbioni, C. Bartoletti, S. Fanali, M.A. Raggi, J. Chromatogr. A 1051 (2004) 253.
[2] Q. Sun, S.V. Olesik, J. Chromatogr. B 745 (2000) 159.
[3] T. Romiguieresa, F. Pehourcqb, M. Matogaa, B. Begaudb, C. Jarry, J. Chromatogr. B 775 (2002) 163.
[4] P.E. Morgan, J. Tapper, E.P. Spencer, J. Chromatogr. B 798 (2003) 211.
[5] A.R. Chaves, S.M. Silva, R.H.C. Queiroz, F.M. Lancas, M.E.C. Queiroz, J. Chromatogr. B 850 (2007) 295.
[6] X. Hong1, Y. Yao1, S. Hong1, C. Lei, Chromatographia 68 (2008) 65.
[7] Z.D. Zhai, Y.P. Shi, T. Wang, Anal. Chim. Acta 550 (2005) 123.
[8] F.J.M. de Santana, E.J. Cesarino, P.S. Bonato, J. Chromatogr. B 809 (2004) 351.
[9] F.J.M. de Santana, A.R.M. Oliveira, P.S. Bonato, Anal. Chim. Acta 549 (2005) 96.
[10] F.J.M. de Santana, V.A.P. Jabor, E.J. Cesarino, V.L. Lanchote, P.S. Bonato, J. Sep. Sci. 33 (2010) 268.
[11] F.J.M. de Santana, P.S. Bonato, Anal. Chim. Acta 606 (2008) 80.
[12] Z. Bosakova, E. Curınova, E. Tesarova, J. Chromatogr. A 1088 (2005) 94.
[13] Chirobiotic Handbook, fifth ed., Advanced Separation Technologies, USA, 2004.
[14] ICH, Guideline Q2A, Text on Validation of Analytical Procedures. International Conference on Harmonization. http://www.ich.org, 1994.
[15] K.W. Phinney, L.A. Jinadu, L.C. Sander, J. Chromatogr. A 857 (1999) 285.
[16] K.M. Fried, P. Koch, I.W. Wainer, Chirality 10 (1998) 484.