

## A New Validated Differential Scanning Calorimetric Procedure for Monitoring the Less Active *R,S* Isomer of Ethambutol Dihydrochloride in Bulk Drug Samples and Anti-tuberculosis Formulations\*

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**ABSTRACT** A new and simple validated differential scanning calorimetric (DSC) procedure was developed for quantitation of the less active *R,S* diastereoisomer of ethambutol dihydrochloride in bulk drug samples and marketed anti-tuberculosis formulations. The procedure involves determination of the enthalpy associated with polymorphic transitions, appearing at 42 °C and 77 °C for *R,S* and *S,S* isomers, respectively. Suitable equations were derived and could be successfully used to determine the relative extents of the two isomers in commercial products. The unwanted *R,S* form was found to be present in more than half of the tested drug substances and products and in some products constituted 100% of the active. This suggests that an appropriate test procedure and relevant limits should be included in *USP–NF* and other international compendia to control the inactive isomer in ethambutol dihydrochloride and also in anti-tuberculosis fixed-dose combination products that contain the drug.

### INTRODUCTION

Ethambutol dihydrochloride (EB2HCl) can exist in three different diastereoisomeric forms: *S,S*; *R,S*; and *R,R*; designated in this paper as SS-EB2HCl, RS-EB2HCl, and RR-EB2HCl, respectively. SS-EB2HCl is therapeutically active, but RS-EB2HCl is 16 times less active, and RR-EB2HCl is completely inactive (1). The synthesis of EB2HCl is carried out using (+)-2-amino-1-butanol as the starting material; hence the process primarily yields a mixture of SS-EB2HCl and RS-EB2HCl without the presence of RR-EB2HCl. RS-

EB2HCl is removed by taking advantage of its low solubility in a number of solvents, but it still can be present in the SS-EB2HCl form of the drug. Incidentally, there is no procedure given in any pharmacopeia to control or determine the extent of RS-EB2HCl in EB2HCl or its products. Only the range of optical rotation values is prescribed to control quality or for assay of the active SS-EB2HCl. The compendial tests (2–5) are listed in *Table 1*, along with their anticipated limitations.

**Table 1. Pharmacopoeial evaluations of the isomeric quality of EB2HCl (2–5)**

Pharmacopoeia	Test	Postulated drawback of the respective test
<i>United States Pharmacopoeia (USP)</i>	Specific rotation between +6.0° and 6.7° of 10% (w/v) solution at 25 °C and 100 mm	1. The wide range of the specific rotation may allow up to 10% of ( <i>R,S</i> ) form as an impurity 2. Not applicable in formulations
<i>Indian Pharmacopoeia (IP)</i>	Same as above	Same as above
<i>Japanese Pharmacopoeia (JP)</i>	Specific rotation between +5.5° and 6.1° of 10% (w/v) solution at 20 °C and 200 mm	Same as above
<i>British Pharmacopoeia (BP)</i>	Assay procedure based on optical activity	1. Not applicable in formulations 2. Low sensitivity of the procedure

Fortunately, a few reports exist in the literature for the determination of RS-EB2HCl. These include a study done in early seventies by Ferrari and Graber (6), who used differential thermal analysis (DTA) for the detection and quantitation of the *meso* (or optically inactive) isomer. Several years later Varshney et al. (7) reported the use of more precise differential scanning calorimetry (DSC) for the purpose. The latter procedure was based on quantitation of an endotherm at 178 °C attributed to solid–solid interaction (eutectic formation) between

RS-EB2HCl and SS-EB2HCl. Although the procedure was novel, its use was severely restricted because quantitation of RS-EB2HCl was limited to the range of 0–5%. At higher concentrations of RS-EB2HCl, the endotherm at 178 °C merged with the melting endotherm of EB2HCl at ~200 °C. Following this report, another procedure appeared in the literature and involved chromatographic separation of diastereoisomers of EB2HCl using a chiral column (8). This procedure did not become popular, perhaps due to high costs and restricted commercial availability of the chromatographic column.

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Lately, Rubin-Preminger et al. (9–10) explored the polymorphic behaviour of RS-EB2HCl and SS-EB2HCl (Figure 1) using a host of instrumental techniques, including variable-temperature solid-state carbon-13 nuclear magnetic resonance (NMR), variable-temperature powder X-ray diffraction (XRD), differential scanning calorimetry (DSC), and optical microscopy. That both RS-EB2HCl and SS-EB2HCl forms of the drug exist independently in two polymorphic states was confirmed through careful comparison of unit cell parameters of single-crystal XRD and packing diagrams, wherein large differences were observed between the bond angles and both lengths of the region surrounding C3. The same were also proved through Rietveld fitting of powder XRD parameters and chemical shifts for C3 in the carbon-13 solid state-NMR spectra of more than 2 ppm. These techniques also indicated that phase transitions were enantiotropic and fully reversible. The latter were also indicated by the appearance of differences in torsion of additional endotherms in DSC for RS-EB2HCl and SS-EB2HCl at 42 °C and 77 °C, respectively.

The occurrence of characteristic endotherms in DSC below the melting point of EB2HCl at ~200 °C indicated the possibility of developing a simple and specific procedure for quantifying RS-EB2HCl in the presence of SS-EB2HCl. This was attempted, with development and validation of the procedure. It was subsequently applied to the analysis of RS-EB2HCl in bulk drug samples and commercial products containing EB2HCl. The results suggested a need for a specific procedure and limits for the unwanted isomer in compendial monographs on EB2HCl API and formulations containing the drug.

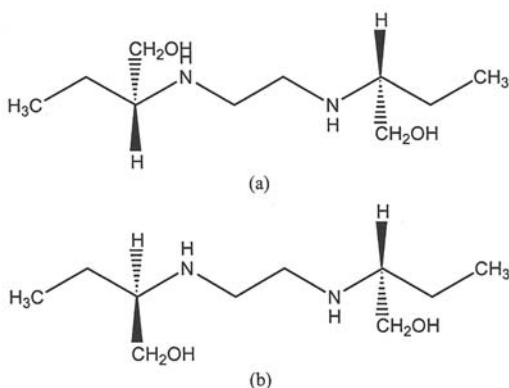


Figure 1. Chemical structure of SS-EB2HCl (a) and RS-EB2HCl (b)

## MATERIALS AND PROCEDURES

### Materials

The APIs were procured directly from the manufacturers, and formulations were procured from local market. SS-EB2HCl was received as a gift sample from Themis Medicare, Vapi, Gujarat, India. RS-EB2HCl was extracted from a marketed formulation that was determined during an initial study to contain only this form. Calibration standards for DSC (indium and zinc) were procured from Mettler Toledo (Schwerzenbach, Switzerland). All solvents used for extraction were of HPLC grade.

## Equipment

DSC analyses were performed using a Mettler Toledo (Schwerzenbach, Switzerland) 821e instrument controlled by STAR software version 5.21. Thermogravimetric analysis (TGA) was performed using a model 851e instrument from the same manufacturer. FTIR spectra of the samples were obtained on an Impact 410 spectrophotometer (Nicolet, WI, USA) equipped with OMNIC analyzing software. Optical rotation and specific rotation were monitored using an analytical polarimeter (model 55, Rudolph Research, Hackettstown, NJ, USA). Ultrapure water was obtained from a purification unit (Elga Ltd., Bucks, England). Rotavapor from Buchi (Flawil, Switzerland) was used for solvent evaporation.

The HPLC system consisted of a DGU-14A degasser module, FCV-10AL<sub>VP</sub> flow control valve, LC-10AT<sub>VP</sub> pump, SIL-10AD<sub>VP</sub> auto injector, CTO-10AS<sub>VP</sub> column oven, SPD-M10A<sub>VP</sub> photodiode array (PDA) detector, and an SCL-10A<sub>VP</sub> system controller; data were acquired and processed using CLASS-VP software version 6.13 (all from Shimadzu, Kyoto, Japan). A Zorbax XDB CN-SB (150 × 4.6 mm, particle size 5µm) column (Agilent Technologies, Wilmington, DE, USA) was used for the chromatographic study. Other devices employed were a pH meter (MA 235, Mettler Toledo GmbH, Schwerzenbach, Switzerland), sonicator (Branson, Ultra-Sonic Corporation, Danbury, CT, USA), analytical balance (AG 135, Mettler Toledo, Greifensee, Switzerland), and autopipettes (Eppendorf, Hamburg, Germany).

### Initial DSC Studies

The first studies were carried out to determine if the characteristic DSC endotherms for RS- and SS-EB2HCl at 42 °C and 77 °C appeared and resolved in all types of samples, whether bulk drugs or the formulations. For this, the uncoated formulations were powdered directly, but the coating of coated tablets was removed manually, followed by powdering to a uniform size suitable for DSC analysis. In some samples, interference was linked to the presence of excipients, for which solvent extraction was carried out. The extraction involved dispersal of the powder in acetonitrile, filtration of the suspension, and collection of filtrate, followed by drying on a rotavapor. The dried filtrate was further washed with acetone to remove solvent-soluble impurities. After samples were redried, thermograms were recorded between 25 and 250 °C at a rate of 10 °C/min.

### Isolation and Characterization of RS-EB2HCl

During initial studies one of the single-drug tablet formulations of EB2HCl showed a polymorphic endotherm at 42 °C without any transition at 77 °C. Studies showed that it contained only RS-EB2HCl. The tablet was powdered and extracted with water, followed by filtration and drying in order to obtain the pure drug sample. The isolated material was characterized using DSC, polarimetry, FTIR, and TGA. The FTIR spectrum was taken using the conventional KBr pellet procedure. The specific rotation was determined taking a 10% solution of the isolated substance in a 10-cm sample holder. DSC studies were performed in aluminium crucibles at a heating

rate of 10 °C/min from 25 to 250 °C under nitrogen purging (20 mL/min). TGA studies were conducted under conditions similar to those for DSC.

### Study of Stability of Polymorphic Transitions

A heating–cooling–heating cycle study was carried out to determine the stability and reproducibility of the two enantiotropic transitions at 42 °C and 77 °C. The isolated RS-EB2HCl and available pure SS-EB2HCl were independently subjected to thermal cycles of 25–100–25–100 °C at a heating/cooling rate of 10 °C/min.

### Optimization of Sample Weight and Heating Rate

The two isomer samples were subjected to DSC studies. The samples were weighed in the range of 2–7 mg and were heated between 25 to 250 °C at a rate of 10 °C/min. Subsequently, a 50:50 mixture of the two forms was subjected to heating from 25 to 250 °C at the rates of 5, 10, and 20 °C/min.

### Quantitative Procedure Development and Validation

The selectivity and dependence on concentration of the endothermic transitions at 42 °C and 77 °C were determined by taking mixtures of SS-EB2HCl and RS-EB2HCl in various ratios from 0 to 100%. Precision was determined by carrying out multiple studies on the same day ( $n=6$ ), followed by studies on two subsequent days to establish intra- and interday variability. This investigation was done also on marketed formulations to evaluate the applicability of the procedure in real situations.

### Determination of the Extent of RS-EB2HCl in Bulk Drug Samples and anti-TB Formulations

For these studies the samples were treated in a manner similar to that reported in “Initial DSC Studies,” and thermograms were recorded again, focusing on enthalpy values.

### Applicability of USP HPLC Procedure to Separate RS-EB2HCl and SS-EB2HCl

The HPLC procedure described in *United States Pharmacopoeia (USP)* for the assay of EB2HCl in single-drug and four-drug fixed-dose combination tablets was evaluated in terms of its ability to separate RS-EB2HCl and SS-EB2HCl. The test conditions, including column, were the same as prescribed by *USP (11)*. RS-EB2HCl alone was injected, followed by RS-EB2HCl spiked with SS-EB2HCl. The chromatographic resolution between the two injections was compared, and the peak purity was checked in both cases.

## RESULTS AND DISCUSSION

### Preliminary DSC Investigations

The preliminary DSC studies on bulk drug samples and formulations (*Table 2*) showed polymorphic endotherms at 42 °C and 77 °C for RS-EB2HCl and SS-EB2HCl, respectively. This was in line with the values reported by Rubin-Preminger et al. (*10*). Some of the investigated samples had single polymorphic transition, and others contained both of them. The extraction step was able to remove interfering substances and yielded well-resolved endotherms of interest at 42 °C and 77 °C. The nature of the thermograms obtained for the tested samples are shown in *Figure 2*.

**Table 2. Bulk drugs and formulations selected for preliminary DSC studies**

Bulk drug/formulation	Code	Manufacturing Date	Expiry Date
Bulk drug	BD-1	January 2003	December 2008
Bulk drug	BD-2	September 2004	August 2009
Single drug tablets	SD-1	October 2003	August 2007
Single drug tablets	SD-2	April 2005	March 2008
Two-drug FDC* tablets	FDC-2/1	April 2004	March 2008
Two-drug FDC tablets	FDC-2/2	February 2005	January 2008
Three-drug FDC tablets	FDC-3/1	March 2004	February 2006
Three-drug FDC tablets	FDC-3/2	July 2004	June 2007
Four-drug FDC tablets	FDC-4/1	January 2004	May 2006
Four-drug FDC tablets	FDC-4/2	May 2005	April 2007

\* FDC = fixed-dose combination

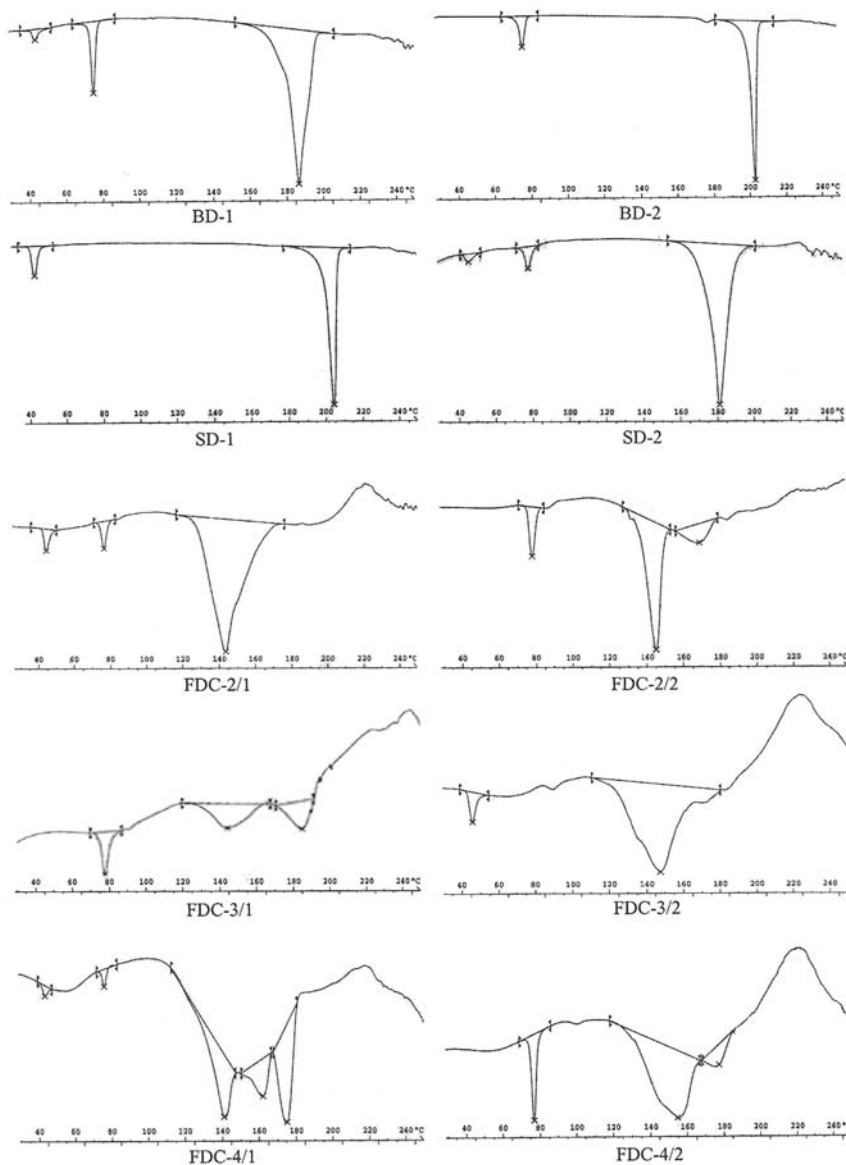


Figure 2. DSC thermograms of selected bulk drug samples and formulations. Key: Sample codes are described in *Table 2*

### Characterization of RS-EB2HCl

The DSC thermogram of isolated SS-EB2HCl showed a single transition at 77 °C and a melting endotherm at 201 °C (*Figure 3a*) in comparison to the characteristic polymorphic transition at 42 °C and the melting endotherm at 202.9 °C shown by pure RS-EB2HCl (*Figure 3b*). The small right shift of melting endotherm of RS-EB2HCl with respect to SS-EB2HCl was in line with the known behaviour for diastereoisomers of EB2HCl (9–10). This is an unusual outcome and is opposite to usual findings with chirally mixed diastereoisomers (12).

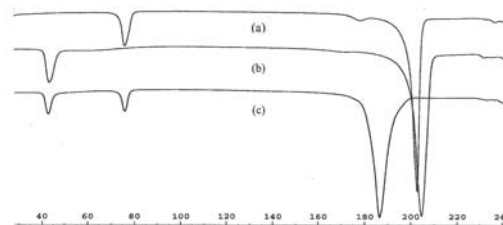


Figure 3. DSC thermogram of SS-EB2HCl (a), RS-EB2HCl (b), and a 50:50 (w/w) combination of the two isomers (c)

The specific rotation values obtained for SS-EB2HCl and RS-EB2HCl were  $6.79 \pm 0.24$  and  $0.29 \pm 0.02$ , respectively. The little specific rotation value for the isolated RS-EB2HCl compared to that for SS-EB2HCl showed that the former was virtually devoid of optical activity. The FTIR spectra of RS-EB2HCl was superimposable on that of SS-EB2HCl (*Figure*



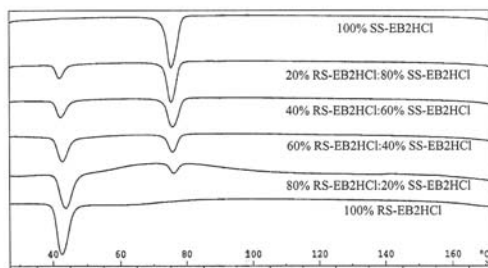


Figure 7. Changes in DSC thermograms at 42 °C and 77 °C for various relative concentrations of RS-EB2HCl and SS-EB2HCl

### Method Validation

The data and regression parameters in *Table 3* showed good linearity between enthalpy and the relative percentage of RS-EB2HCl and SS-EB2HCl in the range of 0 to 100%. The correlation coefficients were >0.99. Also, good intra- and inter-day precision was established (RSD <1.5%) even though the investigation involved a complex formulation containing four anti-TB drugs (*Table 4*).

**Table 3. Enthalpy data for RS-EB2HCl and SS-EB2HCl and regression parameters for the linearity curves**

Ratio of RS-EB2HCl:SS-EB2HCl	Enthalpy, J/g	
	Endotherm at 42 °C for RS-EB2HCl	Endotherm at 77 °C for SS-EB2HCl
0:100	0	25.85
2:98	0.52	25.09
10:90	2.21	22.40
20:80	4.67	19.88
30:70	7.16	17.49
40:60	9.10	15.44
50:50	11.53	11.35
60:40	13.78	9.24
70:30	16.58	6.90
80:20	18.48	3.96
90:10	21.51	2.22
100:0	23.10	0
Regression parameters for the linearity curve	$y = 0.2338x - 0.0366,$ $r^2 = 0.9992$	$y = 0.2605x - 0.7506,$ $r^2 = 0.9968$
Correction factor	1.114	1

### Quantitation of Relative Percentages of RS and SS-EB2HCl

$$\%RS-EB2HCl = [CF \times M / (CF \times M + D)] \times 100 \quad [1]$$

As shown from the data in *Table 3*, the slopes for the linearity curves were different for RS-EB2HCl and SS-EB2HCl. Accordingly, a correction factor (CF) was derived, given by  $Slope_{[SS-EB2HCl]} / Slope_{[RS-EB2HCl]}$ . The same was applied to the following equations for the calculation of the relative percentages of RS and SS-EB2HCl in a mixture:

$$\%SS-EB2HCl = [D / (CF \times M + D)] \times 100 \quad [2]$$

where *M* and *D* are the phase transition enthalpies for RS-EB2HCl and SS-EB2HCl at 42 °C at 77 °C, respectively.

**Table 4. Precision studies\***

Validation parameter	Formulation	%RS-EB2HCl ± SD, %RSD	%SS-EB2HCl ± SD, %RSD
Intraday precision	SD2	32.50 ± 0.44, 1.36	67.39 ± 0.44, 0.66
	FDC-4/1	34.80 ± 0.15, 0.43	65.20 ± 0.15, 0.23
Interday precision	SD2	33.14 ± 0.25, 0.77	66.86 ± 0.25, 0.38
	FDC-4/1	35.20 ± 0.53, 1.50	64.80 ± 0.53, 0.82

\* Key: Codes for SD2 and FDC-4/1 are according to *Table 2*; SD = standard deviation; RSD = relative standard deviation

Table 5 lists the relative percent values of RS-EB2HCl and SS-EB2HCl in two bulk drug samples and eight marketed formulations.

**Table 5. Respective content of isomers in tested bulk drugs and formulations**

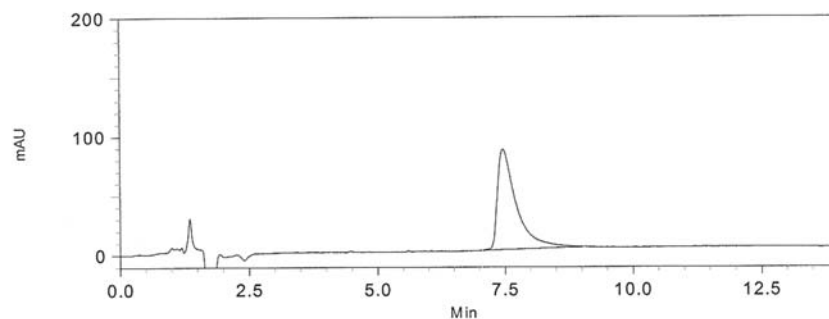
Sample Code	%SS-EB2HCl	%RS-EB2HCl
BD-1	100.00	0.00
BD-2	80.84	19.16
SD-1	0.00	100.00
SD-2	66.80	33.20
FDC-2/1	52.05	47.95
FDC-2/2	100.00	0.00
FDC-3/1	0.00	100.00
FDC-3/2	100.00	0.00
FDC-4/1	64.80	35.20
FDC-4/2	100.00	0.00

### Extent of the Presence of RS-EB2HCl in Bulk Samples and Marketed Formulations

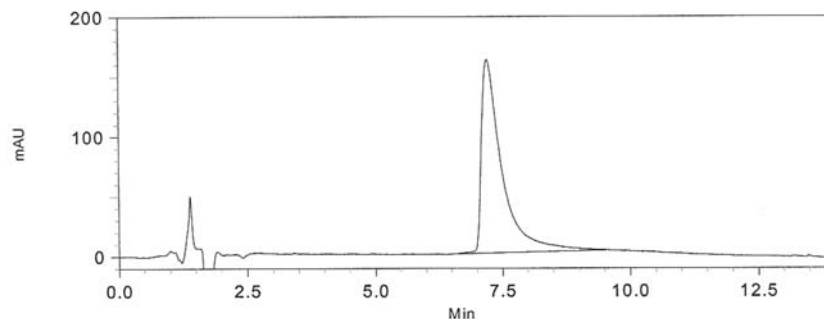
As evident from Table 5, six of the ten bulk drugs and products evaluated in this study contained from ~20% to 100% of the less active RS-EB2HCl isomer, indicating a need to limit this inactive related substance in compendial monographs.

### Evaluation of the Procedure in USP 29

The chromatographic profiles obtained for RS-EB2HCl alone and in combination with SS-EB2HCl, are shown in Figure 8. Evidently, a single peak appeared in both the cases at almost the same retention time, and both were pure by photodiode array purity analysis. The current USP procedure for assay of EB2HCl was unable to distinguish between RS-EB2HCl and SS-EB2HCl isomeric forms. This inability to differentiate between the two isomers, with resulting consequences for the efficacy of drug products to treat life-threatening disease, justifies the need for inclusion of an alternative procedure in USP monographs on EB2HCl drug substance and products.



(a)



(b)

Figure 8. HPLC chromatograms of RS-EB2HCl (a) and combination of SS-EB2HCl and RS-EB2HCl (b)

### Status in Other Pharmacopoeias

As indicated in *Table 1*, the procedures in the *Indian Pharmacopoeia (IP)* and the *Japanese Pharmacopoeia (JP)* are almost similar to that in *USP*. Therefore, these pharmacopoeias would also be unable to differentiate between the diastereomers. The optical activity–based assay procedure given in the *British Pharmacopoeia (BP)* features the additional disadvantage that it cannot be applied to coloured formulations that contain rifampicin along with EB2HCl. This drawback should be considered along with the procedure's low sensitivity. Therefore, the proposed DSC method is recommended for adoption by all compendia for the detection and control of RS-EB2HCl.

### CONCLUSION

A procedure was developed for monitoring the relative presence of the isomers of EB2HCl (RS-EB2HCl and SS-EB2HCl) in commercial bulk drug samples and formulations containing the drug. This was done by utilizing the polymorphic transitions in DSC thermograms at 42 °C and 77 °C for RS-EB2HCl and SS-EB2HCl, respectively. The procedure was validated and proven to be precise and simple and required no sample preparation in most cases. It was also applied successfully to complex two-, three-, and four-drug fixed-dosage combinations containing EB2HCl along with rifampicin, isoniazid, and/or pyrazinamide. The applicability of the procedure to coloured anti-TB formulations containing rifampicin is of particular benefit because optical rotation procedures, which are usually employed for monitoring isomers, fail when the test samples are coloured.

The study strongly suggests the need to control the unwanted isomeric related substance in compendial monographs of EB2HCl, as well as in single-drug and fixed-dosage combination formulations containing the drug. Because *USP* is the first compendium to include monographs for anti-TB FDCs, it may take the lead to add a suitable test to monitor and control the presence of RS-EB2HCl in monographs of EB2HCl and products. The same should be done even by other compendia,

keeping in view that tuberculosis is a global emergency and good-quality drugs are necessary to control the dreaded disease.

### REFERENCES

1. Wilkinson RG, Shepard RG, Thomas JP, Baughn C. Stereospecificity in a new type of synthetic anti-tuberculosis agent. *J Am Chem Soc.* 1961;83:2212–2213.
2. USP. *USP 29–NF 24*. Rockville, MD: United States Pharmacopoeial Convention, Inc; 2006:859.
3. *IP 1996*. New Delhi, India: Ministry of Health and Family Welfare; 1996:297.
4. *JP 14*. Tokyo, Japan, Ministry of Health, Labour, and Welfare; 2001:460.
5. *BP 2005*. London, UK, British Pharmacopoeial Commission; 2005:764.
6. Ferrari H, Graber DG. Thermal properties of ethambutol (myambutol): the detection of the (*R,S*) isomer in the presence of ethambutol by differential thermal analysis. *Microchem J.* 1971;16:5–13.
7. Varshney L, Sharma G, Gopal NGS. Determination of the *meso* isomer in dextro ethambutol hydrochloride by differential scanning calorimetry. *Ind Drugs.* 1988;26:117–119.
8. Blessington B, Beiraghi A. A method for the quantitative enantioselective HPLC analysis of ethambutol and its stereoisomer. *Chirality.* 1992;3:139–144.
9. Rubin-Preminger JM, Bernstein J, Haris RK, Evans IR, Ghi PY. (*R,S*)-ethambutol dihydrochloride: variable-temperature studies of a dimorphic system with very similar packing. *J Pharm Sci.* 2004;93:2810–2819.
10. Rubin-Preminger JM, Bernstein J, Haris RK, Evans IR, Ghi PY. Variable temperature studies of a polymorphic system comprising two pairs of enantiotopically related forms: (*S,S*)-ethambutol dihydrochloride. *Cryst Growth Des.* 2004;4:431–439.
11. USP. *USP 29–NF 24*. Rockville, MD: United States Pharmacopoeial Convention, Inc.; 2006:860,1922,1923.
12. Jacques J, Collet A, Wilen SH. Enantiomers, racemates, and resolutions. New York: John Wiley & Sons; 1981:94,253–259,238–339.