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# Chromatographic separation of (*E*)- and (*Z*)-isomers of entacapone and their simultaneous quantitation in human plasma by LC–ESI-MS/MS

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ABSTRACT

A selective, sensitive and high throughput liquid chromatography–tandem mass spectrometry (LC–ESI-MS/MS) method has been developed and validated for the chromatographic separation and quantitation of (*E*)-entacapone and (*Z*)-entacapone in human plasma. Sample clean-up involved liquid–liquid extraction (LLE) of both the isomers and carbamazepine used as internal standard from 500 μL of human plasma. Both the analytes were chromatographically separated with a resolution factor of 3.0 on a Gemini C18 (50 mm × 4.6 mm, 5 μm particle size) analytical column using 1% formic acid and methanol (50:50, v/v) as the mobile phase. The selectivity factor ($\alpha$) of the column for the separation was 2.0, based on the capacity factors of 2.6 and 1.3 for (*E*)- and (*Z*)-isomers respectively. The parent → product ion transitions for both the isomers (*m*/*z* 306.1 → 233.0) and IS (*m*/*z* 237.3 → 194.2) were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM) and positive ion mode. The method was validated over the concentration range of 24.3–6076 ng/mL and 23.8–5960 ng/mL for (*E*)-entacapone and (*Z*)-entacapone respectively. Matrix effect was assessed by post-column analyte infusion experiment and the process/extraction efficiency found was 94.3% and 89.3% for (*E*)- and (*Z*)-isomers respectively. The method was successfully applied to a pivotal bioequivalence study in 36 healthy human subjects after oral administration of 200 mg (*E*)-entacapone tablet formulation under fasting conditions.



## 1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative disorders found in the elderly [1]. Levodopa, the most effective drug in the treatment of PD is degraded by catechol-*O*-methyltransferase (COMT) enzyme in the peripheral nervous system, resulting in dyskinesias and motor fluctuations. Inhibition of COMT activity can prolong the action of levodopa and reduce fluctuations in response. Entacapone [(*E*)-2-cyano-*N*,*N*-diethyl-3-(3,4-dihydroxy-5-nitrophenyl)propenamide], a selective and reversible COMT inhibitor is extensively used as an adjunct to levodopa/dopa decarboxylase inhibitors in the treatment of Parkinson's disease [2,3]. It enhances levodopa absorption and reduces its peripheral metabolism, leading to greater levodopa availability to the brain. The presence of nitro group at the position *ortho* to the hydroxyl group is critical for entacapone's potency and ability to inhibit COMT. Entacapone is rapidly absorbed after oral administration with a bioavailability of 35%, secondary to first-pass clearance and is almost completely metabolized before extraction (0.1–0.2% of dose unchanged in urine). The drug exhibits a linear pharmacokinetic profile over the dose range of 5–800 mg. Entacapone is 98% plasma protein bound in vitro-mainly to albumin over the concentration range of 0.4–50 μg/mL [4,5]. Wikberg et al. [6] have identified four metabolites of entacapone in humans and eight metabolites in rat urine. The glucuronides of entacapone (70%) and its (*Z*)-isomer (25%) were the major urinary metabolites formed. The (*Z*)-isomer of entacapone and two less abundant urinary metabolites were obtained by reduction of the side chain carbon-carbon double bond. The (*Z*)-isomer was the only phase I metabolite found in addition to entacapone in human plasma.

Few bioanalytical methods are reported for the separation and quantitation of (*E*)- and (*Z*)-isomers of entacapone in biological matrices. Hynnila et al. [7,8] have reported the analysis of nitrocatechol-type glucuronides in urine samples by liquid chromatography–tandem mass spectrometry and capillary electrophoresis–tandem mass spectrometry respectively. The limit of detection of 3-*O*-glucuronides of (*E*)-entacapone and (*Z*)-

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entacapone in both the methods was 100 ng/mL. In another method [9] they have described the quantitation of entacapone glucuronide isomers in rat plasma by on-line coupled restricted access media column using column switching liquid chromatography–tandem mass spectrometry. The chromatographic separation was achieved in a run time of 20 min and the quantitation was possible over a wide dynamic range of 0.0025–100 μg/mL. Two methods based on micellar electrokinetic capillary chromatography have been developed by Lehtonen et al. [10,11] for the separation of two main urinary metabolites of entacapone in humans. The electropherograms in both these methods required 18 min for complete resolution of 3-*O*-glucuronide isomers (*E* and *Z*) of entacapone. The limit of quantitation was 2 μg/mL for both analytes and the assay was linear in the range of 2–100 μg/mL and 2–150 μg/mL respectively. Ramakrishna et al. [12] have developed a selective HPLC-UV method to quantify (*E*)-entacapone in human plasma. The linear dynamic range of 25–2500 ng/mL was achieved by employing 1.0 mL plasma volume for processing. A reversed phase HPLC method with amperometric detection was presented by Karlsson and Wikberg [13] to analyze entacapone and its *Z*-isomer in human plasma and urine. The method was highly sensitive with an LLOQ of 10 ng/mL, but the extraction procedure was time consuming with long chromatographic run time.

The objective of the present study was to separate both the isomers chromatographically and to develop an LC–ESI-MS/MS method for their quantitation in human plasma. The proposed method exhibited excellent performance in terms of sensitivity, selectivity, ruggedness and efficiency (4.0 min per sample) due to cleaner extracts, with simplicity of sample preparation. It ensured the estimation of both the isomers up to 24 h with desired accuracy and precision for bioequivalence study in human volunteers.

## 2. Experimental

### 2.1. Chemicals and materials

Reference standards of (*E*)-entacapone (99.8%) and (*Z*)-entacapone (92.5%) were procured from Unichem Laboratories Ltd. (Mumbai, India) and carbamazepine (99.2%, IS) was purchased from Samex Overseas (Surat, India). HPLC grade methanol and acetonitrile were obtained from Mallinckrodt Baker, (S.A.de C.V. Mexico). Guaranteed reagent grade formic acid, HPLC grade dichloromethane and diethyl ether were obtained from Merck Specialties Pvt. Ltd., (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system procured from Millipore (Bangalore, India). Blank human plasma was procured from Supratech Micropath (Ahmedabad, India) and was stored at −20 °C until use.

### 2.2. Liquid chromatographic conditions

A Waters Acquity UPLC system (Milford, MA, USA) consisting of binary solvent manager, sample manager and column manager was used for setting the reverse-phase liquid chromatographic conditions. The separation of (*E*)-entacapone, (*Z*)-entacapone and carbamazepine (IS) was performed on a Phenomenex analytical column, Gemini C18 [50 mm × 4.6 mm (length × inner diameter), with 5 μm particle size] and was maintained at 35 °C in column oven. The mobile phase consisted of 1% formic acid and methanol in 50:50 (v/v) ratio. For isocratic elution, the flow rate of the mobile phase was kept at 1.0 mL/min with 90% flow splitting; flow directed to the ISP interface was equivalent to 100 μL/min. The total chromatographic run time was 4.0 min. The sample manager temperature was maintained at 5 °C and the pressure of the system was 1600 psi.

### 2.3. Mass spectrometric conditions

Ionization and detection of analyte and IS was carried out on a triple quadrupole mass spectrometer, WATERS, Quattro Premier XE (Milford, MA, USA) equipped with electro spray ionization and operating in positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor parent → product ion (*m*/*z*) transitions for entacapone and its (*Z*)-isomer was 306.1 → 233.0 and 237.3 → 194.2 for IS respectively (Fig. 1).

The source dependent parameters maintained for entacapone, its (*Z*)-isomer and carbamazepine were capillary: 4.00 kV; extractor: 3.00 V; RF lens: 0.0 V; source temperature: 100 °C; desolvation temperature: 400 °C; cone gas flow: 50 ± 10 L/h; desolvation gas flow: 850 ± 10 L/h. The optimum values for compound dependent parameters (MRM file parameters) like cone voltage and collision energy set were 38 V and 18 eV for the analytes and 30 V and 17 eV for IS respectively. The dwell time was set at 200 ms. Mass Lynx software version 4.1 was used to control all parameters of UPLC and MS.

### 2.4. Standard stock, calibration standards and quality control sample preparation

The standard stock solution of 1 mg/mL of (*E*)-entacapone and (*Z*)-entacapone was prepared by dissolving requisite amount in methanol. Calibration standards and quality control (QC) samples were prepared by spiking (2% of total volume of blank plasma) blank plasma with stock solution. Calibration curve standards were made at 24.3, 48.6, 122, 304, 760, 1519, 3038 and 6076 ng/mL and 23.8, 47.7, 119, 298, 745, 1490, 2980 and 5960 ng/mL concentrations respectively while quality control samples were prepared at three levels, viz. 5431 ng/mL (HQC, high quality control), 543 ng/mL (MQC, middle quality control) and 69.5 ng/mL (LQC, low quality control) for (*E*)-isomer and 5320 ng/mL (HQC, high quality control), 532 ng/mL (MQC, middle quality control) and 68.1 ng/mL (LQC, low quality control) for (*Z*)-isomer. Stock solution (1 mg/mL) of the internal standard was prepared by dissolving 10 mg of carbamazepine in 10 mL of methanol. An aliquot of 150 μL of this solution was further diluted to 25.0 mL in the same diluent to obtain a solution of 6.0 μg/mL. All the solutions (standard stock, calibration standards and quality control samples) were stored at 2–8 °C until use.

### 2.5. Protocol for sample preparation

Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 500 μL of spiked plasma sample, 50 μL internal standard was added and vortexed for 20 s. Further, 100 μL of 5% formic acid in water was added and vortex mixed for 20 s. To these samples, 2.5 mL of extraction solvent (diethyl ether:dichloromethane; 80:20, v/v) was added and the samples were extracted on extractor at 32 × *g* for 5 min. Centrifugation of the samples was done at 3200 × *g*, for 5 min at 10 °C. 2.0 mL of supernatant was separated and evaporated to dryness under nitrogen at 35 ± 0.5 °C. The dried samples were reconstituted with 100 μL of mobile phase and 7 μL was used for injection in the chromatographic system.

### 2.6. Method validation

The method validation was performed as per the USFDA guidelines [14]. System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of (*E*)-entacapone, (*Z*)-entacapone and internal standard at the start

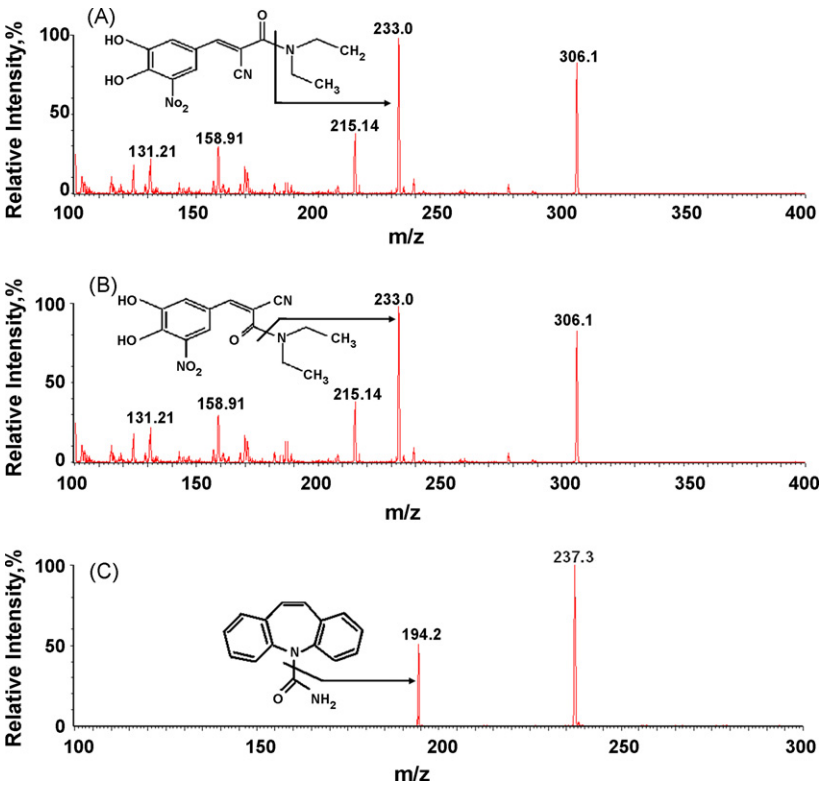


**Fig. 1.** Product ion mass spectra of (A) (*E*)-entacapone (*m*/*z* 306.1 → 233.0, scan range 100–400 amu) (B) (*Z*)-entacapone (*m*/*z* 306.1 → 233.0, scan range 100–400 amu) and (C) carbamazepine, IS (*m*/*z* 237.3 → 194.2, scan range 100–300 amu) in positive ionization mode.

of each batch during the method validation. The carryover effect of the autosampler was evaluated by injecting a sequence of injections solutions of aqueous standard ((*E*)- and (*Z*)-isomers), mobile phase, standard blank and extracted standard ((*E*)- and (*Z*)-isomers) equivalent to highest standard in the calibration range. As per the acceptance criteria, the response in blank should not be greater than 20% of LLOQ response [15].

The linearity of the method was determined by analysis of five linear curves containing eight non-zero concentrations. The ratio of area response for isomers to IS was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression. The lowest standard on the calibration curve was accepted as the lower limit of quantitation (LLOQ), if the analyte response was at least five times more than that of drug free (blank) extracted plasma. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±15.0%.

The selectivity of the method towards endogenous plasma matrix components was assessed in twelve batches (7 normal of K3 EDTA plasma, 2 haemolysed, 2 lipidemic and 1 heparinised) of blank human plasma. This was done to estimate the extent to which endogenous plasma components contribute towards interference at the retention time of analytes and IS. The cross talk of MRM for analytes and IS was checked using highest standard on calibration curve and working solution of IS.

For determining the intra-day accuracy and precision, replicate analysis of plasma samples of (*E*)- and (*Z*)-isomers was performed on the same day. The run consisted of a calibration curve and six replicates of LLOQ, LQC, MQC and HQC samples. The inter-day accuracy and precision were assessed by analysis of three precision and accuracy batches on three consecutive validation days. The precision of the method was determined by calculating the percent coefficient of variation (%CV) for each level. The deviation at each concentration level from the nominal concentration was expected to be within ±15.0% except LLOQ, for which it should be within ±20.0%.

Ion suppression/enhancement effects on the MRM LC–MS/MS sensitivity were evaluated by the post column analyte infusion experiment. A standard solution containing (*E*)- and (*Z*)-isomers (at HQC level) and IS was infused post column into the mobile phase at 10 μL/min employing in-built infusion pump. Aliquots of 7 μL of extracted control plasma were then injected into the column by the autosampler and MRM LC–MS/MS chromatogram was acquired for the analytes and IS. Any dip in the baseline upon injection of double blank plasma (without IS) would indicate ion suppression, while a peak at the retention time of analytes and IS indicates ion enhancement.

The relative recovery, matrix effect and process efficiency were assessed as recommended by Matuszewski et al. [16]. All three parameters were evaluated at HQC, MQC and LQC levels in six replicates. Relative recovery (RE) was calculated by comparing the mean area response of extracted samples (spiked before extraction) to that of unextracted samples (spiked after extraction) at each QC level. The recovery of IS was similarly estimated. Absolute matrix effect (ME) was assessed by comparing the mean area response of unextracted samples (spiked after extraction) with mean area of neat standard solutions. The overall 'process efficiency' (%PE) was calculated as (ME × RE)/100. The assessment of relative matrix effect was based on direct comparison of the MS/MS responses (peak areas) of the analytes spiked into extracts originating from different lots of plasma. The variability in these responses, expressed as %CV was considered as the measure of relative matrix effect.

Stability experiments were carried out to examine the analyte stability in stock solutions and in plasma samples under different conditions. Short term stability at room temperature and long term stability of spiked solution stored at −50 °C was assessed by comparing the area response of stability sample of analyte and IS with

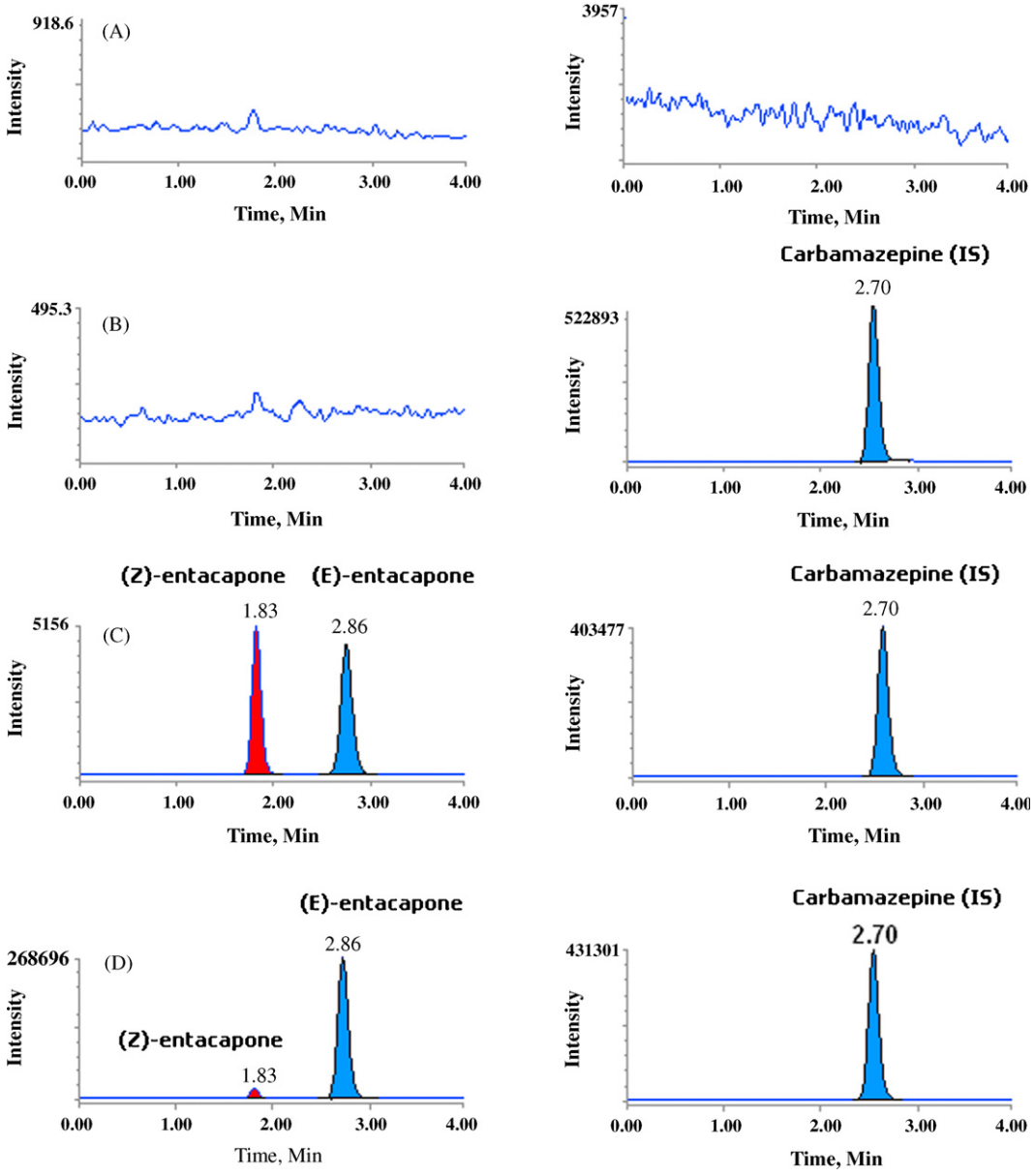


**Fig. 2.** MRM ion-chromatograms of (A) double blank plasma (without IS), (B) blank plasma with IS, (C) (*E*)-entacapone and (*Z*)-entacapone at LLOQ (*m*/*z* 306.1 → 233.0) and IS (D) real subject sample at 0.7 h after administration of 200 mg dose of (*E*)-entacapone.

the area response of sample prepared from fresh stock solutions. The solutions were considered stable if the deviation from nominal value was within ±10%. Autosampler (wet extract) stability, bench top stability, dry extract stability and freeze–thaw stability were performed at LQC and HQC, using six replicates at each level. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed quality control samples was within ±15%.

To authenticate the ruggedness of the proposed method, it was done on two precision and accuracy batches. The first batch was analyzed by two different analysts while the second batch was analyzed on different columns.

Dilution integrity experiment was conducted by diluting the stock solution prepared as spiked standard at concentration of 13325 ng/mL and 12240 ng/mL for (*E*)- and (*Z*)-isomers respectively. The precision and accuracy for dilution integrity standards at 1/5th (2665 ng/mL and 2448 ng/mL) and 1/10th (1332.5 ng/mL and 1224 ng/mL) dilutions for (*E*)- and (*Z*)-isomers respectively were determined by analyzing the samples against calibration curve standards.

### 2.7. Bioequivalence study design

The design of the study comprised of a randomized, open label, balanced, single dose, two treatments, two periods, two sequences, and crossover comparative bioequivalence study of 200 mg (*E*)-entacapone under fasting condition. Each volunteer was judged to be in good health through medical history, physical examination and routine laboratory tests. Written consent was taken from all the volunteers after informing them about the objectives and possible risks involved in the study. An independent ethics committee constituted as per Indian Council of Medical Research (ICMR) approved the study protocol. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA [17]. A single oral dose of 200 mg drug was given to the volunteers with 240 mL of water. Blood samples were

collected at 0.0 (pre-dose), 0.16, 0.33, 0.5, 0.67, 0.83, 1.0, 1.16, 1.33, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 10.0, 12.0, 16.0 and 24.0 h after oral administration of the dose for test and reference formulation in labeled K3 EDTA-vacuettes. Plasma was separated by centrifugation (3200 × *g*, 10 °C, 10 min) and kept frozen at −50 °C until analysis. During study, volunteers had a standard diet while water intake was free.

## 3. Results and discussion

### 3.1. Method development

Chromatographic resolution of (*E*)-entacapone, (*Z*)-entacapone and IS was initiated under isocratic conditions to obtain adequate response, sharp peak shape and a short analysis time. Thus, separation was tried using various combinations of methanol/acetonitrile, acidic buffers and additives like formic acid on four different reversed-phase columns with 5 μm particle size viz. ACE Cyano (150 mm and 250 mm × 4.6 mm), Chromolith RP-18 (100 mm × 4.6 mm), Kromasil (50 mm and 100 mm × 4.6 mm), and Gemini C-18 (50 mm × 4.6 mm) to find the optimal column that produced the best sensitivity, efficiency and peak shape. The analytes showed poor separation and reproducibility for proposed linear range except for Gemini C-18 column that offered superior peak shape, baseline separation, desired linearity and reproducibility. The mobile phase consisting of 0.1% formic acid in water and methanol (50:50, v/v) ratio and having pH around 3.0–3.5 were found most suitable for eluting (*E*)-entacapone, (*Z*)-entacapone and IS at 2.86, 1.83 and 2.70 min respectively. The capacity factors, which describe the rate at which the analytes migrate through the column, were 2.6 and 1.2 respectively for both the isomers based on the dead time of 0.8 min. The selectivity factor ($\alpha$) of the column for the chromatographic separation of (*E*)- and (*Z*)-isomers was 2.0. The number of theoretical plates obtained for (*E*)-, (*Z*)-isomer and IS were 2100, 1340 and 2900 respectively with a resolution factor of 3.0 between the analytes. Also, the reproducibility of retention times for the analytes, expressed as %CV was ≤1% for 100 injections on the same column.

The inherent selectivity of MS/MS detection was also expected to be beneficial in developing a selective and sensitive method. The present study was conducted using ESI as the ionization source as it gave high intensity for the (*E*)-entacapone, (*Z*)-entacapone and IS, and a good linearity in regression curves. The intensity found was much higher in the positive mode for both the analytes and IS as they have similar sites for protonation. Also, the use of formic acid, pH 3.00 in the mobile phase further augmented the response of protonated precursor $[M+H]^+$ ions at *m*/*z* 306.1 for analytes and 237.3 for IS in the Q1 MS full scan mass spectra. The most consistent and abundant product ions at *m*/*z* 233.0 (elimination of diethylamine) and 194.2 were found by applying 18 and 17 eV collision energy for the isomers and IS respectively. A dwell time of 200 ms was adequate for the analytes and IS and no cross talk was observed between their MRMs.

Initially, the extraction of (*E*)-entacapone, (*Z*)-entacapone and IS was carried out via protein precipitation with common solvents like acetonitrile, methanol and acetone, but the sensitivity (especially for (*E*)-entacapone) and reproducibility were poor, in all the solvents with frequent clogging of the column, which required online flushing of the column. Liquid–liquid extraction technique was also tested to isolate the drugs from plasma using diethyl ether, dichloromethane, methyl tert butyl ether and isopropyl alcohol (alone and in combination) as extracting solvents. However, the recovery was inconsistent with some ion suppression (greater than 15% CV) in most of these solvent systems. Further, use of 100 μL of 5% formic acid prior to extraction in diethyl ether: dichloromethane (80:20, v/v) gave consistent recoveries for the analytes, especially at the LLOQ level with minimum matrix interference. A general internal standard was used to minimize any analytical variation due to solvent evaporation, integrity of the column and ionization efficiency of analytes. Carbamazepine was used as an internal standard (IS) in the present study, which had similar chromatographic behavior and was quantitatively extracted with the proposed extraction procedure. Also, there was no effect of IS on analyte recovery, sensitivity or ion suppression.

### 3.2. System suitability and autosampler carryover

Throughout the method validation, the %CV of system suitability was observed below 4.0% at the retention time of (*E*)-entacapone, (*Z*)-entacapone and the IS. Carryover evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method. There was negligible carryover (≤4% of the LLOQ response) observed during autosampler carryover experiment. No enhancement in the response was observed in double blank after subsequent injection of highest calibration standard (aqueous and extracted) at the retention time of analytes and IS.

### 3.3. Linearity and lower limit of quantification (LLOQ)

The calibration curves were linear over the concentration range of 24.3–6076 ng/mL and 23.8–5960 ng/mL with correlation

**Table 1**
Comparison of intra- and inter-batch precision and accuracy for (*E*)-entacapone and (*Z*)-entacapone.

| QC ID | Nominal concentration (ng/mL) | Intra-batch | | | | Inter-batch | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *n* | Mean concentration observed (ng/mL)[a] | %CV | % Accuracy | *n* | Mean concentration observed (ng/mL)[b] | %CV | % Accuracy |
| (*E*)-entacapone | | | | | | | | | |
| HQC | 5431 | 6 | 5464 | 4.2 | 100.6 | 15 | 5512 | 6.1 | 101.5 |
| MQC | 543 | 6 | 561 | 5.9 | 103.3 | 15 | 554 | 6.5 | 102.0 |
| LQC | 69.5 | 6 | 69.8 | 8.9 | 100.4 | 15 | 70.3 | 7.4 | 101.2 |
| LLOQ QC | 25.0 | 6 | 26.5 | 5.2 | 106.0 | 15 | 26.7 | 4.2 | 106.8 |
| (*Z*)-entacapone | | | | | | | | | |
| HQC | 5320 | 6 | 5488 | 4.2 | 103.2 | 15 | 5528 | 6.2 | 103.9 |
| MQC | 532 | 6 | 566 | 5.0 | 106.4 | 15 | 565 | 6.0 | 106.2 |
| LQC | 68.1 | 6 | 70.5 | 9.5 | 103.5 | 15 | 71.5 | 7.7 | 105.0 |
| LLOQ QC | 24.5 | 6 | 26.1 | 3.6 | 106.5 | 15 | 26.3 | 3.5 | 107.3 |

CV, coefficient of variance; *n*, total number of observation.
[a] Mean of 6 replicates at each concentration.
[b] Mean of 15 replicates at each concentration.

coefficient $r \geq 0.9984$ and $r \geq 0.9985$ for (*E*)-entacapone and (*Z*)-entacapone respectively. The equations for means ($n = 5$) of five calibration curves for (*E*)-entacapone was $y = 0.0005x - 0.0010$, while for (*Z*)-entacapone it was $y = 0.0004x - 0.0007$. The standard deviation value for slope, intercept and correlation coefficient ($r$) observed were 0.00006, 0.00005; 0.0002, 0.0002; 0.0006 and 0.0002 for (*E*) and (*Z*)-isomers respectively. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 93.4 to 104.9% and 0.7 to 7.6% for (*E*)-entacapone and from 92.1 to 104.7% and 0.6 to 6.4% for (*Z*)-entacapone respectively. The lowest concentration (LLOQ) in the standard curve for both the isomers was measured at a signal-to-noise ratio (S/N) of ≥100.

### 3.4. Selectivity, accuracy and precision

To establish the selectivity of the method for interference due to endogenous plasma components from haemolysed, lipidemic, heparinised and K3 EDTA blank plasmas, the % change in the area ratio (analyte/IS) at LLOQ level was within 4–6%, while the precision (%CV) in their measurement varied from 2.0 to 4.5%. Representative MRM ion chromatograms in Fig. 2 of (A) extracted blank human plasma (double blank), (B) blank plasma fortified with IS ($m/z$ 237.3 → 194.2), (*E*)-entacapone and (*Z*)-entacapone at LLOQ ($m/z$ 306.1 → 233.0) and a real subject sample at 0.7 h demonstrates the selectivity of the method. The extraction procedure together with mass detection gave very good selectivity for the analysis of both the isomers and IS in the blank plasma. No endogenous interferences were found at the retention times of analytes and IS.

The intra- and inter-batch precision and accuracy were established from validation runs performed at HQC, MQC, LQC and LLOQ QC levels (Table 1). The intra- and inter-batch precision ranged from 4.2 to 8.9% for (*E*)-entacapone and 3.5 to 9.5% for (*Z*)-entacapone. The accuracy values were within 100.4–107.3% for both the analytes in intra- and inter-batches.

### 3.5. Matrix effect, ion suppression, recovery and stability results

Matrix effect may be defined as a composite of some undesirable effects that originate from a biological matrix. These components may result in ion suppression/enhancement, decrease/increase in sensitivity of analytes over a period of time, increased baseline, imprecision of data, drift in retention time and distortion or tailing of a chromatographic output. Result of post-column infusion experiment in Fig. 3 indicates no ion suppression or enhancement at the retention time of analytes and IS as evident from the flat baseline. The observed ion suppression at 0.4 min did not influence quantita-

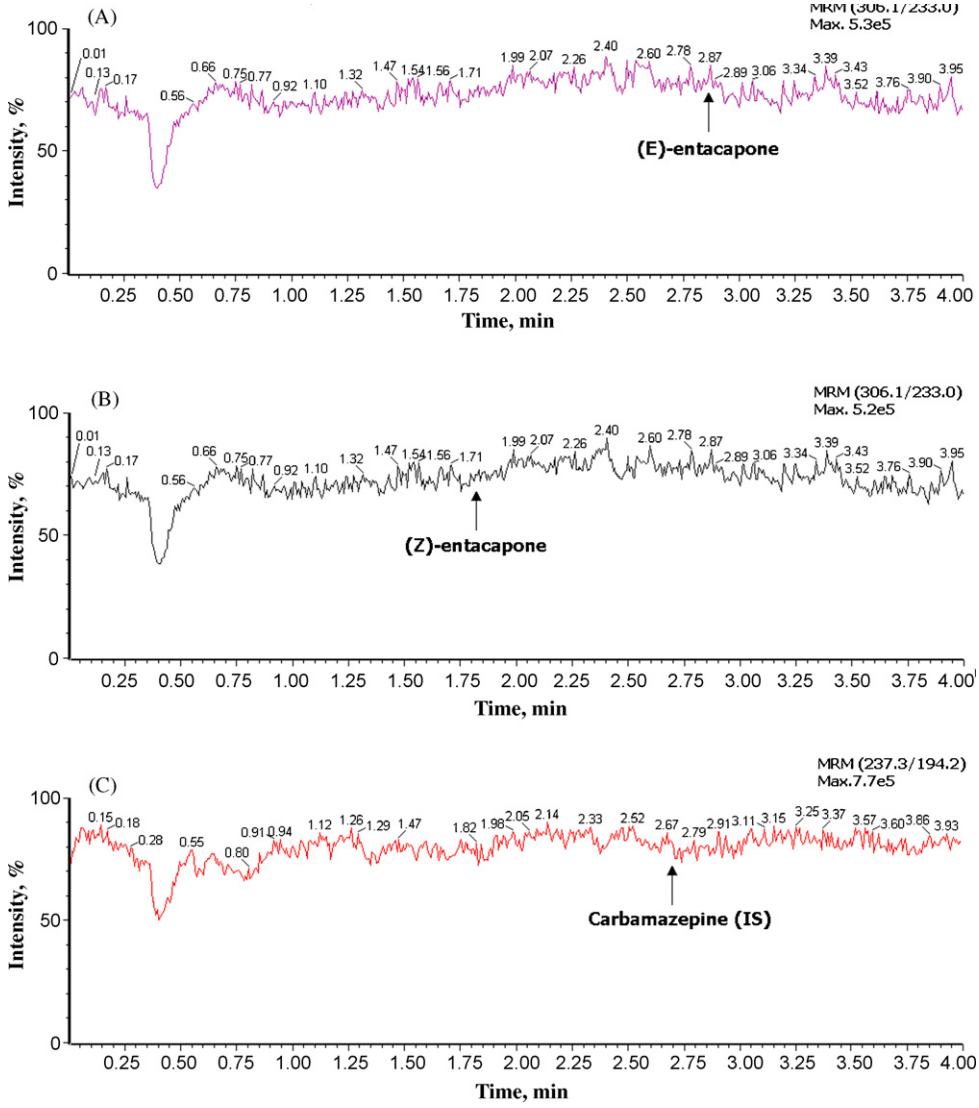


**Fig. 3.** Post column analyte infusion experiment MRM LC–MS/MS chromatograms for (A) (*E*)-entacapone, (B) (*Z*)-entacapone and (C) carbamazepine (IS).

**Table 2**
Absolute matrix effect, relative recovery and process efficiency for (*E*)-entacapone and (*Z*)-entacapone.

| Analyte | *A*[a] (%CV)[b] | *B*[c] (%CV)[b] | *C*[d] (%CV)[b] | Absolute matrix effect (%ME)[e] | Relative recovery (%RE)[f] | Process efficiency (%PE)[g] |
|---|---|---|---|---|---|---|
| LQC | | | | | | |
| (*E*)-entacapone | 6569 (2.01) | 6871 (1.98) | 6335 (2.71) | 104.6 | 92.2 | 96.4 |
| (*Z*)-entacapone | 6416 (2.10) | 6653 (2.05) | 5881 (2.82) | 103.7 | 88.4 | 87.1 |
| MQC | | | | | | |
| (*E*)-entacapone | 54450 (1.85) | 54341 (1.70) | 51787 (1.91) | 99.8 | 95.3 | 95.1 |
| (*Z*)-entacapone | 53878 (1.66) | 49945 (1.42) | 48347 (1.85) | 92.7 | 96.8 | 89.7 |
| HQC | | | | | | |
| (*E*)-entacapone | 589386 (1.30) | 562864 (1.37) | 538098 (1.26) | 95.5 | 95.6 | 91.3 |
| (*Z*)-entacapone | 576573 (1.83) | 568501 (1.63) | 524726 (1.40) | 98.6 | 92.3 | 91.0 |

[a] Mean area response of six replicate samples prepared in mobile phase (neat samples).
[b] Coefficient of variation.
[c] Mean area response of six replicate samples prepared by spiking in extracted blank plasma.
[d] Mean area response of six replicate samples prepared by spiking before extraction.
[e] $B/A \times 100$.
[f] $C/B \times 100$.
[g] $C/A \times 100 = (ME \times RE)/100$.

**Table 3**
Relative matrix effect in six different lots of human plasma (spiked after extraction) for (*E*)-entacapone and (*Z*)-entacapone at LLOQ level ($n = 6$).

| Lot no. | (*E*)-entacapone | | (*Z*)-entacapone | |
|---|---|---|---|---|
| | Mean area response | %CV | Mean area response | %CV |
| 1 | 6650 | 3.1 | 6400 | 1.9 |
| 2 | 6595 | 2.9 | 6465 | 2.7 |
| 3 | 6690 | 3.5 | 6510 | 1.1 |
| 4 | 6610 | 3.9 | 6435 | 3.5 |
| 5 | 6584 | 1.2 | 6428 | 1.5 |
| 6 | 6595 | 1.7 | 6545 | 1.8 |

tion or sensitivity. The relative recovery, absolute matrix effect and process efficiency data at LQC, MQC and HQC levels is presented in Table 2. The recovery for IS in human plasma was 93.9%. Further, the extent of matrix effect in different lots of plasma (spiked after extraction) was within the acceptable limit as evident from the precision (%CV) values in Table 3.

Stock solutions for short term stability of (*E*)-entacapone, (*Z*)-entacapone and IS were stable at room temperature for minimum period of about 7 h and between 2 and 8 °C for about 7 days. (*E*)-entacapone and (*Z*)-entacapone in control human plasma (bench top) at room temperature was stable at least for 8 h at ambient temperature and for minimum of three freeze and thaw cycles. Autosampler stability of the spiked quality control samples maintained at 5 °C was determined up to 11.5 h. Long term stability of the spiked quality control samples stored at −50 °C was determined up to 65 days. The accuracy values for different stability experiments in plasma are shown in Table 4.

### 3.6. Ruggedness and dilution integrity

The results of ruggedness study for (*E*)-entacapone and (*Z*)-entacapone were well within the acceptance limit of 15% in precision and 85.0–115.0% in mean accuracy. The precision and accuracy values for both experiments at LLOQ, LQC, MQC and HQC levels for (*E*)-entacapone ranged from 2.6 to 7.9% and 99.1 to 104.4% respectively. For (*Z*)-entacapone, the precision and accuracy values observed were within 2.5–8.6% and 97.7–102.9% respectively for all the quality control levels.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentration above the upper limit of quantification (ULOQ), which may be encountered during real subject sample analysis. The precision and accuracy values for 1/5th and 1/10th dilution ranged from 5.0 to 5.6% and 102.1 to 105.1% for (*E*)- and (*Z*)-isomers.

### 3.7. Application of the method in healthy human subjects

The validated method was successfully applied for the assay of (*E*)-entacapone, (*Z*)-entacapone in healthy male Indian volunteers

**Table 4**
Stability of (*E*)-entacapone and (*Z*)-entacapone under various conditions ($n = 3$).

| Storage conditions | (*E*)-entacapone | | | (*Z*)-entacapone | | |
|---|---|---|---|---|---|---|
| | Nominal concentration (ng/mL) | Mean calculated conc. (ng/mL) ± SD | % Mean accuracy | Nominal concentration (ng/mL) | Mean calculated conc. (ng/mL) ± SD | % Mean accuracy |
| Bench top stability | | | | | | |
| HQC | 5431 | 4981 ± 148 | 91.7 | 5320 | 4920 ± 165 | 92.5 |
| LQC | 69.5 | 62.7 ± 2.5 | 90.2 | 68.1 | 62.4 ± 4.6 | 91.6 |
| Freeze & thaw stability | | | | | | |
| HQC | 5431 | 4957 ± 255 | 91.3 | 5320 | 4921 ± 178 | 92.5 |
| LQC | 69.5 | 64.3 ± 1.3 | 92.5 | 68.1 | 66.0 ± 1.4 | 97.0 |
| Dry extract stability | | | | | | |
| HQC | 5431 | 4955 ± 152 | 91.2 | 5320 | 5392 ± 120 | 101.4 |
| LQC | 69.5 | 63.8 ± 0.3 | 91.8 | 68.1 | 67.0 ± 1.8 | 98.4 |
| Wet extract stability | | | | | | |
| HQC | 5431 | 5613 ± 140 | 103.4 | 5320 | 5221 ± 117 | 98.1 |
| LQC | 69.5 | 67.2 ± 1.6 | 96.7 | 68.1 | 62.8 ± 0.7 | 92.2 |
| Long term stability in plasma at −50 °C | | | | | | |
| HQC | 5431 | 5513 ± 151 | 101.5 | 5320 | 5486 ± 136 | 103.1 |
| LQC | 69.5 | 70.3 ± 0.9 | 101.2 | 68.1 | 67.5 ± 2.1 | 99.1 |

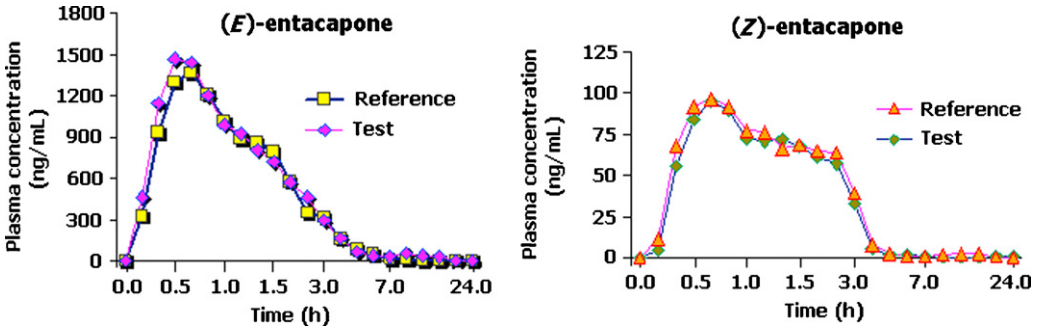


**Fig. 4.** Mean plasma concentration–time profile of (*E*)-entacapone and (*Z*)-entacapone after oral administration of 200 mg (test and reference) (*E*)-entacapone tablet formulation to 36 healthy volunteers.

**Table 5**
Pharmacokinetic parameters (Mean ± SD) of (*E*)-entacapone and (*Z*)-entacapone after oral dose of 200 mg (*E*)-entacapone tablet formulation to 36 healthy human subjects under fasting condition.

| Parameter | (*E*)-entacapone | | (*Z*)-entacapone | |
|---|---|---|---|---|
| | Test | Reference | Test | Reference |
| $C_{max}$ (ng/mL) | 1559 ± 727 | 1509 ± 691 | 105 ± 41 | 103 ± 43 |
| $T_{max}$ (h) | 0.7 ± 0.5 | 0.8 ± 0.4 | 0.7 ± 0.5 | 0.8 ± 0.5 |
| $t_{1/2}$ (h) | 0.8 ± 0.5 | 1.7 ± 1.0 | 2.1 ± 1.0 | 2.0 ± 0.9 |
| $AUC_{0-24\,h}$ (h ng/mL) | 1727 ± 713 | 1566 ± 383 | 98.7 ± 69 | 88.9 ± 36.9 |
| $AUC_{0-inf}$ (h ng/mL) | 1738 ± 363 | 1659 ± 495 | 260 ± 387 | 213 ± 212 |

in the age group of 18–45 years. Fig. 4 shows the plasma concentration vs. time profile of (*E*)-entacapone and (*Z*)-entacapone human subjects under fasting condition. The method was sensitive enough to monitor the (*E*)-entacapone and (*Z*)-entacapone plasma concentration up to 24 h. Approximately 3744 samples including the calibration and QC samples with volunteer samples were run and analyzed during a period of 22 days and the precision and accuracy for calibration and QC samples were well within the acceptable limits. The pharmacokinetic parameters viz. $C_{max}$, $AUC_{0-t}$, $AUC_{0-inf}$, $T_{max}$, and $t_{1/2}$ were calculated for entacapone in test and reference formulations (Table 5). The 90% confidence interval of individual ratio geometric mean for test/reference was within 80–125% for $AUC_{0-t}$, $AUC_{0-inf}$ and $C_{max}$. The mean maximum plasma concentration and $T_{max}$ values for (*E*)-entacapone were comparable with those reported by Ramakrishna et al. [12] in Indian volunteers. Also, the elimination half life of oral (*E*)-entacapone (200 mg) is within the range as cited in previous reports [4,5]. These observations confirm the bioequivalence of the test sample with the reference product in terms of rate and extent of absorption. Further, there were no adverse events during the course of the study.

## 4. Conclusion

The proposed method successfully demonstrates chromatographic separation of (*E*)-entacapone and (*Z*)-entacapone from human plasma with high resolution. The bioanalytical methodology for their simultaneous determination is highly specific, rugged and rapid for therapeutic drug monitoring. The method involved a simple and specific sample preparation by liquid–liquid extraction followed by isocratic chromatographic separation in 4.0 min. The overall analysis time is promising compared to other reported procedures for both the isomers. The established LLOQ and a wide linear dynamic range is adequate to conduct a pharmacokinetic study with 200 mg or higher dose formulations of entacapone in healthy human volunteers.

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