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High-performance liquid chromatography method for the quantification of entacapone in human plasma

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Abstract

A simple, sensitive and selective HPLC method with UV detection (315 nm) was developed and validated for quantitation of entacapone in human plasma, the newest addition to the group of antiparkinsonian agents. Following a single-step liquid–liquid extraction (LLE) with ethyl acetate/*n*-hexane (30/70, v/v), the analyte and internal standard (rofecoxib) were separated using an isocratic mobile phase of 30 mM phosphate buffer (pH 2.75)/acetonitrile (62/38, v/v) on a reverse phase C_{18} column. The lower limit of quantitation was 25 ng/mL, with a relative standard deviation of less than 8%. A linear range of 25–2500 ng/mL was established. This HPLC method was validated with between-batch and within-batch precision of 2.2–4.2% and 1.7–7.8%, respectively. The between-batch and within-batch accuracy was 98.7–107.5% and 97.5–106.0%, respectively. Frequently coadministered drugs did not interfere with the described methodology. Stability of entacapone in plasma was excellent, with no evidence of degradation during sample processing (autosampler) and 30 days storage in a freezer. This validated method is sensitive, simple and repeatable enough to be used in pharmacokinetic studies.

Keywords: Entacapone; HPLC; Human plasma; Pharmacokinetic study

1. Introduction

Entacapone (Fig. 1) is a new adjunct to levodopa therapy in the treatment of Parkinson's disease. The drug is a potent, specific and orally acting peripheral catechol-*O*-methyltransferace (COMT) inhibitor [1].

In current clinical practice, levodopa is almost always given with a peripheral inhibitor of aromatic amino acid decarboxylase (AADC) – benserazide or carbidopa – to prevent peripheral formation of dopamine and to increase levodopa bioavailability [2]. However, in the presence of AADC inhibition, the 3-*O*-methylation of levodopa via COMT becomes a more important metabolic pathway, leading to fast elimination of levodopa and accumulation of its metabolite 3-*O*-methyldopa. COMT inhibitor entacapone reduces the conversion of levodopa to 3-*O*-methyldopa, resulting in an increased area under the concentration curve (AUC) for plasma levodopa [3] and therefore improve the bioavailability of levodopa in the brain.

Entacapone is rapidly absorbed after oral administration of a single dose and mean maximum plasma concentrations of 1160–1500 ng/mL are generally reached 0.7–1.3 h after administration of 200 mg dose in patients with Parkinson's disease. The elimination of entacapone is mainly described by two phases: the β-phase represents approximately 90% of elimination and the γ-phase about 10% [1,3–5]. The elimination half-life of oral entacapone 200 mg is 1–2 h [6], which is similar to the elimination half-life of levodopa (≈1.7 h) [7].

A few methods have been reported for the quantification of entacapone and its metabolites in biological fluids [8–11]. Karlsson and Wikberg [8] reported a HPLC method with electrochemical detection for the assay of entacapone in human plasma and urine. Although selective and highly sensitive, assay procedures making use of electrochemical detection is often very time consuming, both in the sample preparation steps and the chromatography. To date, no simple HPLC

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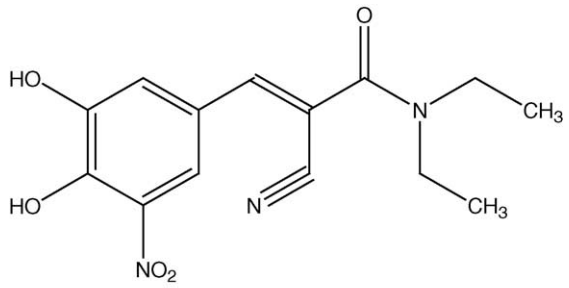
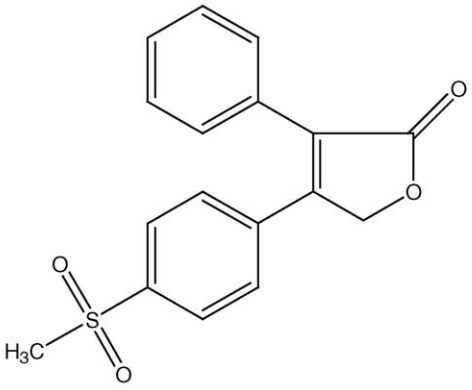


Entacapone



Rofecoxib (I.S.)

Fig. 1. Chemical structures of entacapone and rofecoxib (I.S.).

method with commonly used ultraviolet absorbance detection has been reported for entacapone quantification at therapeutic concentrations in human plasma.

The objective of the present investigation is to establish a fully validated HPLC method with a quantification limit sufficiently low to support pharmacokinetic and bioequivalence studies of entacapone. The method reported in this paper is a simple, accurate HPLC method to quantify the plasma concentration of entacapone with ultraviolet detection using liquid–liquid extraction (LLE). This method is fully validated as per FDA guidelines [12] and the lower limit of quantification (LLOQ) is 25 ng/mL. Additionally, it provides information about the stability of entacapone both in plasma and during sample processing (autosampler), which is a clear advantage for determining a large number of plasma samples for pharmacokinetic and bioequivalence studies in patients and healthy subjects.

2. Experimental

2.1. Chemicals

Entacapone drug substance was obtained from Wockhardt Research Centre (Aurangabad, India). Rofecoxib (internal standard, I.S.) was obtained from Cadila Healthcare Ltd. (Ahmedabad, India). Chemical structures are presented in Fig. 1. HPLC-grade LiChrosolv acetonitrile was from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate, di-potassium hydrogen phosphate, ethyl acetate, *n*-hexane and *ortho*-phosphoric acid were purchased from Merck (Worli, Mumbai, India). HPLC Type I water from Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

2.2. Chromatography

The integrated high performance liquid chromatography system (LC 2010C, Shimadzu Corporation, Kyoto, Japan) was equipped with a quaternary pump, a degasser, an autosampler, an injector with a 100-μL loop, a column oven, a UV detector and a data system (Class VP version 6.12). The separation of compounds was made on a symmetry® (Waters corporation, Milford, USA) C_{18} column (5 μm, 250 mm × 4.6 mm, i.d.) at 25 °C temperature. The mobile phase was a mixture of 30 mM phosphate buffer (20 mM potassium dihydrogen phosphate buffer and 10 mM di-potassium hydrogen phosphate buffer; pH adjusted to 2.75 with *ortho*-phosphoric acid)/acetonitrile (62/38, v/v) pumped at a flow-rate of 1.0 mL/min. Detection was set at a wavelength of 315 nm.

2.3. Sample processing

A 1-mL volume of plasma was transferred to a 15-mL glass test tube, and then 25 μL of I.S. working solution (100 μg/mL) was spiked. Hundred microliters of 10% *ortho*-phosphoric acid was added to the mixture and vortex mixed for 10 s. Next a 4-mL aliquot of extraction solvent, ethyl acetate/*n*-hexane (3/7), was added using Dispensette Organic (Brand GmbH, Postfach, Germany). The sample was vortex-mixed for 4 min using vortexer. The sample was then centrifuged using centrifuge for 3 min at 800 × *g*. The organic layer (3-mL) was quantitatively transferred to a 6-mL glass tube and evaporated to dryness using evaporator at 40 °C under a stream of nitrogen. Then, the dried extract was reconstituted in 250 μL of water/acetonitrile (50/50, v/v; diluent) and a 100-μL aliquot was injected into chromatographic system.

2.4. Bioanalytical method validation

2.4.1. Calibration and control samples

Standard stock solutions of entacapone (1 mg/mL) and I.S. (1 mg/mL) were prepared in acetonitrile. The I.S. working solution (100 μg/mL) was prepared by diluting stock solution with water/acetonitrile (50/50, v/v; diluent). Fifty microliters of working solutions were added to 950 μL of drug-free plasma to obtain entacapone concentrations of 25, 50, 100, 200, 500, 1000 and 2500 ng/mL. The quality control samples were prepared in pool, at concentrations of 25 (LLOQ), 75 (low), 1000 (medium) and 2000 ng/mL (high),

as a single batch at each concentration, and then divided in aliquots that were stored in the freezer at below −50 °C until analysis.

A calibration curve was constructed from a blank sample (a plasma sample processed without an I.S.), a zero sample (a plasma processed with I.S.) and seven non-zero samples covering the total range (25–2500 ng/mL), including LLOQ. The calibration curves were generated on five consecutive days. Linearity was assessed by a weighted ($1/x^2$) least squares regression analysis. The calibration curve had to have a correlation coefficient (r^2) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%. At least 67% of non-zero standards should meet the above criteria, including acceptable LLOQ and upper limit of quantitation.

2.4.2. Selectivity

Randomly selected five blank human plasma samples, which were carried through the extraction procedure and chromatographed to determine the extent to which endogenous plasma components may contribute to interference with the analyte or the internal standard. The results were compared with LLOQ (25 ng/mL).

2.4.3. Recovery

Recovery of entacapone was evaluated by comparing the mean peak areas of five extracted low, medium and high quality control samples to mean peak areas of five neat reference solutions (unprocessed). Recovery of rofecoxib (I.S.) was evaluated by comparing the mean peak areas of five extracted quality control samples to mean peak areas of five neat reference solutions (unprocessed) of the same concentration.

2.4.4. Accuracy and precision

Within-batch accuracy and precision evaluations were performed by repeated analysis of entacapone in human plasma. The run consisted of a calibration curve plus five replicates of each LLOQ, low, medium and high quality control samples. Between-batch accuracy and precision were assessed by analysis of samples consisting of a calibration curve and five replicates of LLOQ, low, medium and high quality control samples for entacapone on three separate occasions.

2.4.5. Stability

The short-term stability was examined by keeping replicates of the low and high plasma quality control samples at room temperature for 24 h. Freeze-thaw stability of the samples was obtained over three freeze-thaw cycles, by thawing at room temperature for 2–3 h, refrozen for 12–24 h. Autosampler stability of entacapone was tested by analysis of processed and reconstituted low and high plasma QC samples, which are stored in the autosampler tray for 30 h. Stability of entacapone in human plasma was tested after storage at below −50 °C for 30 days. The stability of standard solutions was also tested at room temperature for 2, 20 h and upon refrigeration (4 °C) for 14 days. For each concentration and each storage condition, five replicates were analyzed in one analytical batch. The concentration of entacapone after each storage period was related to the initial concentration as determined for the samples that were freshly prepared and processed immediately.

3. Results and discussion

3.1. Separation

The chromatographic conditions, especially the composition of mobile phase and its pH, were optimized through several trials to achieve good resolution and symmetric peak shapes of analytes as well as short run time. It was found that a mixture of 30 mM phosphate buffer (pH adjusted to 2.75 with *ortho*-phosphoric acid)/acetonitrile (62/38, v/v) could achieve our purpose.

Fig. 2 shows the representative chromatograms of blank plasma, plasma samples spiked with entacapone at 1000 ng/ml and at LLOQ (25 ng/ml), and plasma sample obtained from a healthy subject after 0.75 h following an oral 200 mg dose of entacapone. The analytes were well separated from co-extracted material under the described chromatographic conditions at retention times of 8.3 and 10.7 min, respectively. The peaks shape were satisfactory and completely resolved one from another at therapeutic concentrations of entacapone. No interference with constituents from the plasma matrix was observed.

3.2. Linearity and sensitivity of the assay

The peak area ratio of entacapone to I.S. in human plasma was linear with respect to the analyte concentration over the range 25–2500 ng/mL. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ($1/x$, $1/x^2$ and $1/\sqrt{x}$). The residuals improved by weighted ($1/x^2$) least-squares linear regression. The best fit for the calibration curve could be achieved with the linear equation $y = mx + c$ with a $1/x^2$ weighing factor. The mean linear regression equation of calibration curve for the analyte was $y = 1268.2\ (\pm 47.1)x + 3.3\ (\pm 2.3)$, where y was the peak area ratio of the analyte to the I.S. and x was the concentration of the analyte. The correlation coefficient (r) for entacapone was above 0.99 over the concentration range used. These calibration curves were suitable for generation of acceptable data for the concentrations of the analyte in the samples during between-batch and within-batch validations (Table 1).

LLOQ was established as 25 ng/mL. The mean response for the analyte peak at the assay sensitivity limit (25 ng/mL) was ≈6.2-fold greater than the mean response for the peak in five blank human plasma samples at the retention time of the analyte.

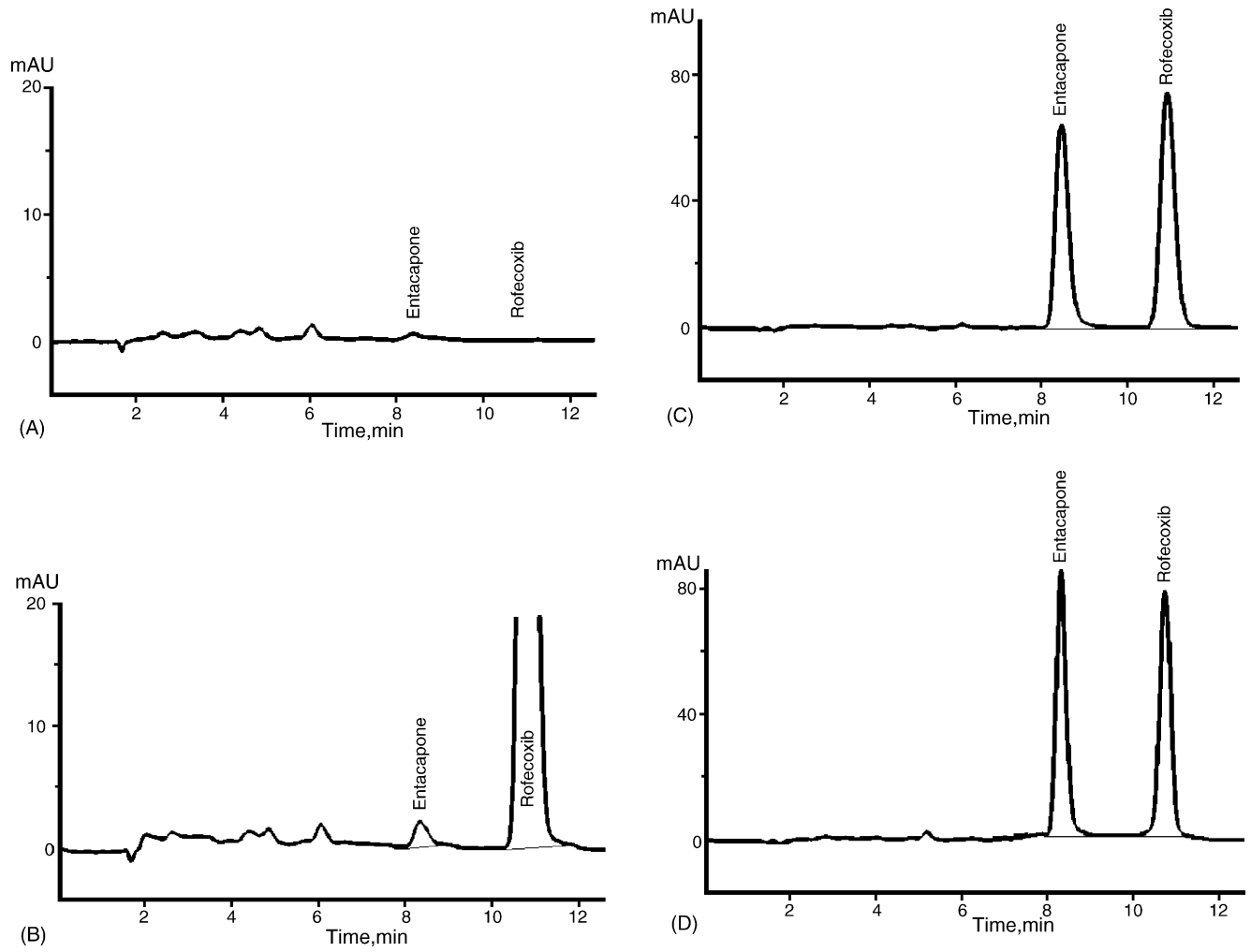


Fig. 2. Chromatograms of (A) blank human plasma; (B) spiked human plasma sample at LLOQ (25 ng/mL); (C) human plasma sample spiked with 1000 ng/mL of entacapone and I.S.; (D) plasma sample from a healthy subject following a 200 mg oral dose of entacapone, the plasma concentration was determined to be 1190 ng/mL for entacapone; approximate retention times: entacapone = 8.3 min; I.S. = 10.7 min.

3.3. Extraction

Liquid–liquid extraction was used for the sample preparation in this investigation. LLE can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column. Six organic solvents, *n*-hexane, ethyl acetate, diethyl ether, dichloromethane, chloroform, *t*-butylmethylether and their mixtures in different combinations and ratios were evaluated. Finally, a mixture of ethyl acetate and *n*-hexane (30/70, v/v) was found to be optimal, which can produce a clean chromatogram for a blank plasma sample and yield the highest recovery for the analyte from the plasma. The extraction recovery of entacapone at low, medium and high quality control samples was 60.4 ± 1.5%, 59.8 ± 2.2% and 61.2 ± 2.4%, respectively. It indicates that extraction recovery of enta-

Table 1
Accuracy and precision of the HPLC method for determining entacapone concentrations in plasma samples

Concentration added (ng/mL)	Within-batch precision ($n = 5$)			Between-batch precision ($n = 3$)		
	Concentration found (mean ± S.D.; ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± S.D.; ng/mL)	Precision (%)	Accuracy (%)
25	25.1 ± 2.0	7.8	100.5	26.4 ± 1.1	4.2	105.6
75	73.1 ± 5.1	6.9	97.5	74.0 ± 1.7	2.3	98.7
1000	1041.8 ± 68.5	6.6	104.2	1063.9 ± 23.3	2.2	106.4
2000	2120.8 ± 37.0	1.7	106.0	2149.6 ± 71.6	3.3	107.5

Table 2
Stability of entacapone in human plasma

Sample concentration (ng/mL) ($n = 5$)	Concentration found (ng/mL)	Precision (%)	Accuracy (%)
Short-term stability for 24 h in plasma			
75	76.7	1.0	102.3
2000	2137.7	2.1	106.8
Three freeze and thaw cycles			
75	78.9	10.4	105.2
2000	2094.0	1.5	104.7
Autosampler stability for 24 h (after extracting and reconstitution)			
75	75.9	14.2	101.2
2000	2121.7	1.7	106.1
30 days stability at <−50 °C			
75	73.1	8.2	97.4
2000	1915.2	2.3	95.7

capone is independent of concentration. The recovery of internal standard, rofecoxib was 69.6% at the concentration used in the assay (100 μg/mL).

3.4. Selectivity

There were no interfering peaks present in five different randomly selected samples of drug free human plasma used for analysis at the retention times of either analyte or internal standard. There was no interference of entacapone and rofecoxib analysis by other potentially co-administered drugs such as OTC drugs, NSAIDs, benserazide, carbidopa and levodopa.

3.5. Precision and accuracy of the assay

The results shown in Table 1 indicate that the assay method is reproducible for replicate analysis of entacapone in human plasma within the same day and also the method is reproducible on different days.

The accuracy values for between- and within-batch studies at the LLOQ and at low, medium and high concentrations of entacapone in plasma were within acceptable limits ($n = 3$) (Table 1).

3.6. Stability

The stock solutions were stable for at least 6 months when stored at 4 °C. The stability experiments were aimed at testing all possible conditions that the samples might experience after collecting and prior the analysis. These were performed as described in Section 2.4.5. All stability results are summarized in Table 2. Three freeze-thaw cycles and 24 h room temperature storage for low and high quality controls samples indicated that entacapone was stable in human plasma under these conditions. QC samples were stable for at least 30 days if stored frozen at approximately −50 °C. Testing of autosampler stability of quality control samples (Table 2) indicated that entacapone is stable when kept in the autosampler for up to 24 h.

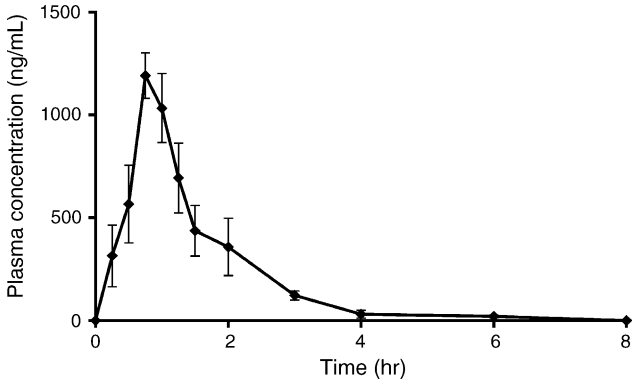


Fig. 3. Mean concentration versus time profiles over 8 h of entacapone in human plasma from six subjects each receiving a single dose of 200 mg entacapone.

3.7. Application to clinical study

The present HPLC method was employed to determine the pharmacokinetic parameters of entacapone in subjects plasma samples of clinical studies. After a single oral dose of 200 mg entacapone to 18 healthy subjects, concentration versus time profiles were constructed for up to 8 h. Fig. 3 shows mean concentration–time profiles of entacapone in six subjects each receiving a 200 mg oral dose of entacapone under fasting conditions.

4. Conclusions

The HPLC/UV method is described employing liquid–liquid extraction for sample preparation and convenient for the quantification of entacapone in human plasma samples and fully validated as per FDA guidelines [12]. The validation data demonstrates good precision and accuracy. The validated method allows quantification of entacapone in the 25–2500 ng/mL range. Because of the straightforward sample preparation procedure, a sample throughput of 60 per 10 h is routinely achieved. In conclusion, this paper describes a

simple and accurate HPLC method for the quantitation of entacapone suitable to monitor plasma concentrations during clinical pharmacokinetic studies in humans.

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