Simultaneous RP-HPTLC Method for Determination of Levodopa, Carbidopa, and Entacapone in Combined Tablet Dosage Form

Deesha B. Gandhi and **Priti J. Mehta***

Key Words

Levodopa
Carbidopa
Entacapone
RP-HPTLC
Method validation
Simultaneous determination

Summary

A simple, rapid, specific, and accurate reverse-phase high-performance thin-layer chromatography (RP-HPTLC) method was developed and validated for simultaneous quantification of levodopa, carbidopa, and entacapone in their combined dosage form. Due to the structural similarity between levodopa and carbidopa, and vast difference in their polarity with that of entacapone, it is very challenging to carry out the simultaneous estimation of all three drugs together. In the developed method, chromatography was performed on TLC plates with precoated silica gel 60 RP-18 F_{254} using acetonitrile–*n*-butanol–water–triethylamine (0.5:9.5:1:0.001, *v*/*v*/*v*/*v*), pH adjusted to 3.6 with *o*-phosphoric acid, as the mobile phase. Densitometric evaluation was performed at 282 nm. The R_F values were 0.46, 0.64, and 0.87 for levodopa, carbidopa, and entacapone, respectively. The polynomial regression data for the calibration plots showed good linear relationship in the concentration range 300–1500 ng per spot for levodopa, 200–1000 ng per spot for carbidopa, and 200–2000 ng per spot for entacapone. The suitability of this HPTLC method for quantitative determination of drugs was proved by validation in accordance with the requirements of the International Conference on Harmonization (ICH) guidelines (Q2B).

1 Introduction

Levodopa (LEV; [(2*S*)-2-amino-3-(3,4-dihydroxyphenyl) propanoic acid]; **Figure 1(a)**) [1–2] is a dopamine precursor used in the management of Parkinsonism. When LEV is administered alone, a major portion of it gets degraded by the peripheral decarboxylase enzyme and only 30% of an orally administered dose reaches the circulation as intact LEV. This circulatory LEV gets metabolized by catechol-*O*-methyltransferase (COMT) enzyme and a very small amount crosses the blood–brain barrier (BBB), which then decarboxylases to dopamine [3]. Carbidopa (CAR; [(2*S*)-3-(3,4-dihydroxyphenyl)-2-hydrazino-2-methylpropanoic acid monohydrate]; **Figure 1(b)**) [1–2] inhibits decarboxylation of peripheral LEV, and therefore administration of CAR with LEV makes more LEV available for transport to the brain [3]. Entacapone (ENT; [(2*E*)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)-*N*,*N*-diethylprop-2-enamide]; **Figure 1(c)**) is a selective and reversible inhibitor of COMT [4]. Thus, it can alter the plasma pharmacokinetics of LEV. When ENT is administered along with LEV and CAR, the plasma level of LEV is greater and more sustained than when administering LEV alone [3]. Thus, the combined formulation of LEV, CAR, and ENT is more effective in the treatment of Parkinsonism for patients experiencing signs and symptoms of end-of-dose 'wearing off' than with therapy of the LEV and CAR combination or LEV alone [5–8].

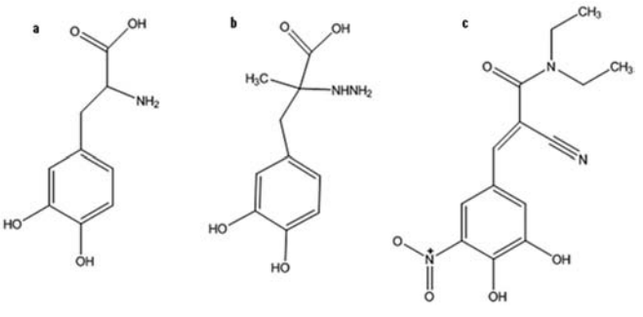

Figure 1

The chemical structure of (a) levodopa, (b) carbidopa, and (c) entacapone.

Individual LEV and CAR as well as a combination of both, are officially recognized in the Indian Pharmacopoeia (IP) and the United States Pharmacopoeia (USP). The IP and USP describe a titrimetric method for the determination of LEV and CAR in their individual tablet formulation and the high-performance liquid chromatography (HPLC) method for the determination of LEV and CAR in their combined tablet formulation [1–2]. The other reported methods for the quantification of LEV, CAR, and ENT individually or in combination with other drugs from dosage forms or in biological fluids include various analytical methods such as spectrophotometry [9–11], fluorometry [12], spectrofluorometry [13], capillary zone electrophoresis with

D.B. Gandhi and P.J Mehta, Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University, S.G Highway, Ahmedabad-382481, Gujarat, India.
E-mail: drpritimehta@nirmauni.ac.in

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amperometry [14], electrochemical detection using modified carbon paste electrode [15], HPLC [16], high-performance thin-layer chromatography (HPTLC) [17], LC stability indicating methods [18–19], and the metabolite identification method [20]. Only one RP-HPLC method has been reported for simultaneous quantification of *in vitro* drug release of LEV, CAR, and ENT [21]. But no method is reported for the simultaneous quantification of LEV, CAR, and ENT in their combined dosage form. Thus, the aim of the present work was to develop a simple, reproducible, and cost-effective analytical method for the simultaneous quantification of LEV, CAR, and ENT in their combined dosage form. This paper describes the development of an RP-HPTLC method and its validation in terms of specificity, linearity, precision, and accuracy, which enhanced the credibility of this analytical method.

2 Experimental

2.1 Materials, Reagents, and Solutions

LEV, CAR, and ENT standards were kindly supplied as a gift by Torrent Pharmaceuticals Limited, Gandhinagar, India. Water purified by a Milli-Q system was used throughout the experimental work. Methanol, *o*-phosphoric acid, and ammonia solution were obtained from Central Drug House (P) Ltd., New Delhi, India. Acetone, acetonitrile, *n*-butanol, 1-octane sulfonic acid sodium salt, chloroform, glacial acetic acid, and triethylamine were from S. D. Fine chemicals, Mumbai, India. All the reagents used were of AR grade. Combined tablet dosage forms were procured from the local market. As LEV and CAR are photosensitive, amber colored volumetric flasks were used for solution preparation.

Stock solutions (1.0 mg mL^{-1}) of the compounds were prepared by dissolving 25 mg of each LEV, CAR, and ENT standard individually in a 25 mL mixture of methanol–0.05 N HCl (1:1, *v*/*v*) in an amber-colored volumetric flask. Working standard solutions (0.1 mg mL^{-1}) were prepared by diluting the stock solutions 1:10 with methanol. A mixture of the drugs was prepared by transferring 1.0 mL of the standard stock into a 10 mL amber volumetric flask and diluting to volume with methanol (the concentration of each drug was 0.1 mg mL^{-1}).

2.2 Instrumentation

TLC aluminum plates precoated with silica gel 60 F_{254} (20 cm × 20 cm, 250 µm thickness) and 60 RP-18 F_{254} (5 cm × 7.5 cm, 250 µm thickness) were obtained from Merck. The HPTLC system consisted of a CAMAG Linomat V semiautomatic spotting device with N_2 flow, a CAMAG 100 µL applicator syringe (Hamilton, Bonaduz, Schweiz), and a CAMAG (10 cm × 10 cm) twin-trough chamber with a stainless steel lid. Densitometry was carried out with a CAMAG TLC Scanner 3 supported by the winCATS software (Version 1.4.2.8121).

2.3 Chromatography

The precoated TLC plates 60 RP-18 F_{254} (5 cm × 7.5 cm, 250 µm thickness) were prewashed with methanol and activated by drying at 50°C for 20 min. Solutions were applied to prewashed TLC plates as 3 mm wide bands, 8 mm from the bottom edge, 10 mm from the side edge, and 7.5 mm apart by means of a CAMAG Linomat-V applicator with the application rate of 15 nL s^{-1}. Chromatograms were run to the solvent front of 50 mm by ascending development in the CAMAG twin-through chamber previously saturated for 25 min with acetonitrile–*n*-butanol–water–triethayamine (0.5:9.5:1:0.001, *v*/*v*/*v*/*v*), pH adjusted to 3.6 ± 0.02 with *o*-phosphoric acid as the mobile phase (run time 22 min). After development, the plates were removed immediately and dried in an oven at 60°C for 2 h. Densitometric scanning at λ = 282 nm was performed with a CAMAG TLC Scanner 3 in the absorbance mode. The silt dimension was kept at 4.0 mm × 0.45 mm and a scanning rate of 20 mm s^{-1} was employed. The chromatograms were integrated using the CAMAG winCATS evaluation software (Version 1.4.2.8121).

2.4 Method Validation

The method was validated for linearity, precision, accuracy, specificity, detection limit (LOD), and quantification limit (LOQ) as per the International Conference on Harmonization (ICH) guidelines [22].

2.4.1 Instrument Precision

Prior to the method validation process, the instrument precision was evaluated in terms of sample application, positioning of

Table 1

Intra-day and Inter-day precision data for LEV, CAR, and ENT by the developed HPTLC method (*n* = 3).

Drug	Quantity [ng] per spot	Intra-day precision		Inter-day precision	
		Mean area ± SD	RSD [%]	Mean area ± SD	RSD [%]
LEV	600	4749.47 ± 74.67	1.57	4839.43 ± 72.54	1.50
	700	5447.33 ± 55.16	1.01	5617.07 ± 103.86	1.85
	800	6942.60 ± 48.92	0.70	6928.37 ± 67.10	0.97
CAR	600	4814.67 ± 25.32	0.53	4853.30 ± 57.40	1.18
	700	5404.10 ± 79.23	1.47	5415.00 ± 81.34	1.50
	800	6256.37 ± 68.26	1.09	6232.17 ± 71.54	1.15
ENT	1000	25724.33 ± 510.53	1.98	26990.63 ± 147.84	0.55
	1200	27948.10 ± 325.14	1.16	27976.37 ± 152.71	0.55
	1400	30372.87 ± 550.81	1.81	30900.23 ± 404.98	1.31

Table 2

Accuracy studies of LEV, CAR, and ENT (n = 3).

Drug	Initial amount [mg]	Fortified amount [mg]	Total amount [mg]	Amount recovered ± SD [mg]	% Recovery ± SD
LEV	100	25	125	126.48 ± 0.90	101.10 ± 0.72
		50	150	150.95 ± 1.05	100.48 ± 0.70
CAR	25	100	125	126.87 ± 1.06	101.33 ± 0.85
		125	150	153.63 ± 1.71	102.35 ± 1.14
ENT	200	50	250	253.55 ± 1.48	101.42 ± 0.59
		100	300	303.27 ± 1.89	101.01 ± 0.63

TLC scanner stage, and repeated scanning of the same spot. A standard solution of LEV of 80 μg mL^{-1} concentration was used. To check the precision of sample application, 10 μL of the solution was repeatedly applied on the plate to make five bands. The plate was developed and analyzed as in Section 2.3. To check the reproducibility of scanning, the same spot was scanned five times and the results were analyzed.

2.4.2 Linearity

For the linearity plot of LEV, 3.0–15.0 μL volumes of the LEV working standard solution (100 μg mL^{-1}, 300–1500 ng), for CAR, 2.0–10.0 μL volumes of the CAR standard solution (100 μg mL^{-1}, 200–1000 ng), and for ENT, 4.0–20.0 μL volumes of the ENT standard solution (100 μg mL^{-1}, 400–2000 ng) were applied to the plate. The plates were developed and analyzed as given in Section 2.3 above. The calibration curves of the peak area *versus* concentration were plotted for all three drugs.

2.4.3 Precision

The intra-day and inter-day precision study data are summarized in **Table 1.** Both studies were performed with a mixed standard solution of 80 μg mL^{-1} LEV, 80 μg mL^{-1} CAR, and 120 μg mL^{-1} ENT in methanol. The intra-day and inter-day studies were performed by injecting three different aliquots of the mixed standard solution in triplicate in a day and on three different days, respectively.

2.4.4 Accuracy

The accuracy (recovery) study data are summarized in **Table 2.** The recovery studies were carried out by adding known quantities of standards at different levels to study the accuracy of the proposed method.

2.4.5 Specificity

The separated chromatographic peaks of all the three drugs were analyzed for peak purity (specificity) by scanning in the range of 200–800 nm with the help of the spectral scanning mode of the winCATs software.

2.5 Analysis of Marketed Tablet Dosage forms

The application of the developed method was evaluated to determine the amounts of LEV, CAR, and ENT in their marketed combined tablet dosage forms with label claims of 100 mg LEV, 25 mg CAR, and 200 mg ENT.

Twenty tablets were weighed, and an amount of tablet powder equivalent to 25 mg of CAR was accurately weighed and transferred to a 100 mL volumetric flask. The CAR standard (100 mg) was added to above tablet powder and dissolved in a 75 mL mixture of methanol–0.05 N HCl (1:1, *v*/*v*). The solution was sonicated for 15 min and then diluted to volume with methanol–0.05 N HCl (1:1, *v*/*v*). It was centrifuged for 15 min at 2500 rpm and then filtered by using a Whatman filter No. 41. The above solution was suitably diluted further with methanol to get a solution containing 50 μg mL^{-1} of LEV, 62.5 μg mL^{-1} of CAR, and 100 μg mL^{-1} of ENT. The plate was activated, and 13 μL of the sample solution was spotted and analyzed as described in Section 2.3.

3 Results and Discussion

3.1 Method Development

HPTLC is the official method in the USP for identification of LEV and CAR in combined tablet dosage form with mobile phase acetone–chloroform–*n*-butanol–glacial acetic acid (GAA)–water (60:40:40:40:35, *v*/*v*/*v*/*v*/*v*), but it requires very long run length (15 cm); the detection of spots also depends on post-derivatization with the ninhydrin reagent. The preliminary trials for the selection of the mobile phase were performed using different compositions of the mobile phase consisting of the aforementioned solvents on TLC silica gel 60 F_{254} plates. But trials on the above mentioned normal phase plate gave inadequate separation, poor chromatographic peak shape, and improper elution of all analytes. Due to the high polarity differences between LEV, CAR (highly polar), and ENT (nonpolar) as well as to the structural similarities between LEV and CAR (CH_3 and NH_2 groups are extra in CAR), separation of LEV, CAR, and ENT simultaneously and at the same time separation of LEV and CAR with good R_F values gave a challenging task. To overcome this problem, TLC plates precoated with silica gel 60 RP-18 F_{254} were used and the mobile phase was optimized by varying its composition. Finally, a mobile phase with *n*-butanol–GAA–water (9.5:0.5:1.5, *v*/*v*/*v*) was found to give good separation but peak shape was not proper. To obtain a good peak shape, triethylamine was added as modifier and acidic pH of the mobile phase was maintained using *o*-phosphoric acid. The optimized mobile phase was acetonitrile–*n*-butanol–water–triethylamine (0.5:9.5:1:0.001, *v*/*v*/*v*/*v*), pH 3.6 ± 0.02 adjusted with *o*-phosphoric acid, which gave symmetric, well-resolved spots of LEV, CAR, and ENT with R_F values of 0.46 ± 0.005, 0.64 ± 0.008, and

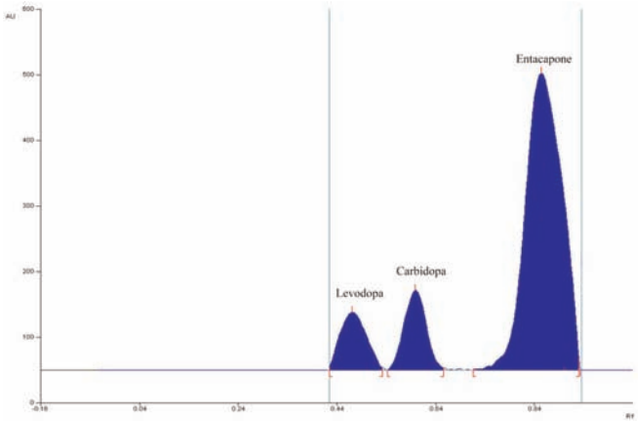

Figure 2

HPTLC chromatogram of LEV (R_F, 0.46 ± 0.005), CAR (R_F, 0.64 ± 0.008), and ENT (R_F, 0.87 ± 0.006) obtained from a standard drug mixture of LEV, CAR, and ENT (each 800 ng per spot).

0.87 ± 0.006, respectively **(Figure 2).** Peak integration of all the drugs was very small when HPTLC plates were scanned after drying at room temperature. So, drying was optimized by drying the plates in an oven at 60°C for different time intervals, and it was found that at 2 h maximum peak intensities of all the drugs were observed. Simultaneous quantification of LEV, CAR, and ENT was performed at 282 nm since all the three drugs exhibit sufficient ultraviolet absorption at this wavelength, and estimation of all drugs was achieved without hampering the sensitivity.

3.2 Validation

3.2.1 Instrument Precision

The results for repeatability of sample application showed that the % RSD was 1.60 for applied spots of the same concentration. For the test on the ability of the instrument to scan the same spots repeatedly, the % RSD was 0.99.

3.2.2 Linearity

The linearity of LEV, CAR, and ENT was assessed to be in the range of 300–1500, 200–1000, and 400–2000 ng per spot, respectively, by determination of slope, intercept, and correlation coefficient. The regression equation and correlation coefficient r^2 of each drug are given in **Table 3.**

3.2.3 Precision

Intra- and inter-day variation in estimation of LEV, CAR, and ENT (Table 1**)** showed that the RSD was always less than 2% during the analysis. These low values of RSD show that the precision of the method is good.

3.2.4 Accuracy

The study of accuracy reveals the positive or negative influence of additives that are usually present in the dosage forms on the quantification parameters. The recovery study data presented in Table 2 indicates that the accuracy of the quantitation of LEV, CAR, and ENT was more than 98%.

3.2.5 Specificity

Typical absorption spectra of LEV, CAR, and ENT are shown in **Figure 3A.** The peak purity of LEV, CAR, and ENT were tested by correlation of the spectra acquired at the peak start (s), peak maximum (m), and peak end (e) positions. Correlation between these spectra confirmed the purity of the LEV peak (correlation $r_{(s,m)}$ = 0.999862, $r_{(m,e)}$ = 0.999437), the CAR peak (correlation, $r_{(s,m)}$ = 0.999542, $r_{(m,e)}$ = 0.998187), and the ENT peak (correlation, $r_{(s,m)}$ = 0.999611, $r_{(m,e)}$ = 0.999133) (Figure 3). The results indicate that in the developed method the excipients did not interfere with the peaks from standard drugs.

3.3 Analysis of Marketed Tablet Dosage Form

The developed and validated RP-HPTLC method was successfully used for the estimation of all the three drugs from its combined tablet dosage form. The percentage amounts of all the three drugs present in all the formulations are given in **Table 4.**

4 Conclusion

The simultaneous estimation of LEV, CAR, and ENT from combined dosage form is a very challenging task due to structural similarity of LEV and CAR and to the wide difference in the polarity of LEV, CAR, and ENT. Here, an RP-HPTLC method was developed and validated as per ICH guidelines for the

Table 3

Linear regression data for analysis of LEV, CAR, and ENT by the developed HPTLC method (n = 3).

Parameter	LEV	CAR	ENT
Linearity range [ng per spot]	300–1500	200–1000	400–2000
Regression equation	$y = 7.407x + 399.9$	$y = 7.236x + 294.3$	$y = 14.75x + 9738$
Correlation coefficient, r^2	0.9997	0.9933	0.9981
LOD [ng per spot]	9.93	10.03	44.75
LOQ [ng per spot]	30.09	30.40	135.59

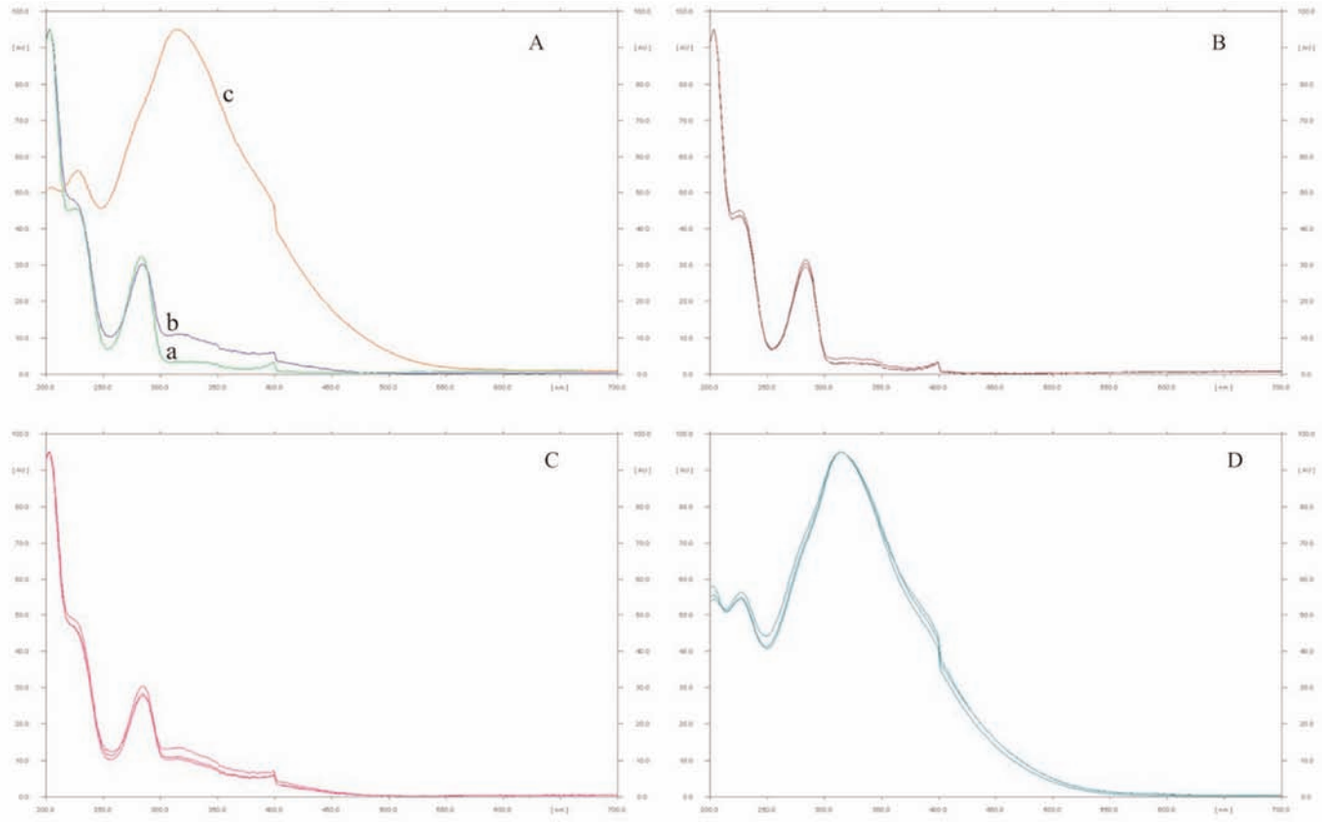

Figure 3

(A) Typical *in situ* absorption spectra of (a) LEV, (b) CAR, and (c) ENT. Peak-purity spectra of (B) LEV, (C) CAR, and (D) ENT extracted from combined tablet dosage form.

Table 4

Simultaneous quantification of LEV, CAR, and ENT in marketed tablet dosage forms by the developed HPTLC method (*n* = 3).

Brand name	Drug	Label claim [mg]	Fortified amount [mg]	Total amount [mg]	Amount recovered ± SD [mg]	% Recovery ± SD
Syncapone	LEV	100	0	100	101.27 ± 1.26	100.77 ± 1.05
	CAR	25	100	125	123.66 ± 0.29	99.08 ± 1.31
	ENT	200	0	200	201.87 ± 1.81	100.66 ± 0.94
Tidomet E-100	LEV	100	0	100	99.79 ± 1.52	99.79 ± 1.52
	CAR	25	100	125	124.41 ± 0.20	99.37 ± 0.81
	ENT	200	0	200	200.20 ± 1.12	100.10 ± 1.06

simultaneous estimation of LEV, CAR, and ENT. A good percentage recovery for all the three drugs showed that the developed method was free from the interference of excipients used in the formulation. The developed method can be successfully used for the simultaneous quantification of LEV, CAR, and ENT in routine quality control analysis of pharmaceutical dosage forms.

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