ISSN: 2348-8948
Vol: 2; Issue: 10

RESEARCH ARTICLE

VALIDATED HPLC AND HPTLC METHOD FOR SIMULTANEOUS QUANTITATION OF ONDANSETRON HYDROCHLORIDE AND PARACETAMOL IN BULK DRUG AND FORMULATION

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Date Received:	Date of Accepted:	Date Published:
21-Sep-2014	29-Sep-2014	5-Oct-2014

Abstract:

Purpose: To develop and validate the HPLC and HPTLC methods for the simultaneous determination of Ondansetron Hydrochloride (OND) and Paracetamol (PARA) in binary mixture. **Methods:** The first method was based on HPTLC separation of the two drugs followed by densitometric measurements of their spots at 286 nm. The separation was carried out on Merck HPTLC aluminium sheets of silica gel 60 F_{254} using toluene: methanol: acetone (6: 2: 2.5, *v/v/v*) as the mobile phase. The second method was based on HPLC separation of the two drugs on the reverse phase PerfectSil-100 ODS-3–C_{18} (250 mm × 4.6 mm, 5.0 µ) from MZ-Analysetechnik GmbH, Germany column and acetonitrile : 0.05 M Ammonium acetate buffer (pH = 6 adjusted with glacial acetic acid) in a ratio of 40:60 as mobile phase. The flow rate was 1 ml/min. **Results:** The linear regression data was used for the regression line in the range of 40–200 ng/spot for OND and 2500-12500 ng/spot for PARA by HPTLC. Quantitation by HPLC was achieved with UV detection at 286 nm based on peak area with linear calibration curves at concentration ranges 2-10 and 125-625 µg/ml for OND and PARA, respectively. **Conclusion:** Both methods have been applied to pharmaceutical formulation without interference of excipients of formulation. Both methods were validated in terms of precision, robustness, recovery, limit of detection and quantitation.

Keywords: Ondansetron Hydrochloride; Paracetamol; HPLC; HPTLC; Validation.

Introduction

OND (Fig. 1a) is chemically (±) 1, 2, 3, 9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl)methyl]-4H-carbazol-4-one. It is a highly specific and selective serotonin 5-HT_3 receptor antagonist. Serotonin activates 5-HT_3 receptor to initiate the vomiting reflex. Ondansetron is an antinauseant and antiemetic agent indicated for the prevention of nausea and vomiting associated with moderately-emetogenic cancer chemotherapy and for the prevention of postoperative nausea and vomiting [1-2]. PARA (Fig. 1b) is chemically N-(4-hydroxyphenyl) acetamide has analgesic and antipyretic effects. Its action is due to inhibition of the cyclooxygenase enzyme and the prostaglandin synthesis in the central nervous system [3] and its direct activity on the centre for the body temperature regulation in the hypothalamus.

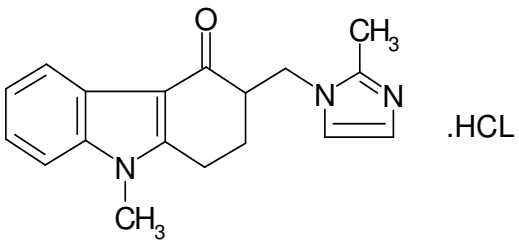

Fig. 1a Ondansetron Hydrochloride

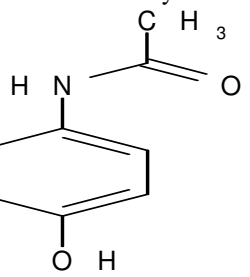

Fig. 1b Paracetamol

Literature review reveals that methods have been reported for analysis of OND by spectrophotometry [4-5], capillary electrophoresis in plasma [6], high-performance liquid chromatography (HPLC) in plasma [7-9] and high-performance thin layer chromatography (HPTLC) [10] and for PARA methods reported are spectrophotometry [11-12], spectrofluorimetry [13-14], colourimetric [15-16], gas chromatography (GC) [17], capillary zone electrophoresis [18], HPLC [19-24] and HPTLC [25-26] either alone or in combination with other drugs.

To date, there have been no published reports about the simultaneous quantitation of OND and PARA by HPLC and HPTLC in bulk drug and in pharmaceutical dosage form. This present study reports for the first time simultaneous quantitation of OND and PARA by HPLC and HPTLC in bulk drug and in pharmaceutical dosage form. The proposed method is validated as per ICH guidelines [27].

2. Experimental

2.1 Materials

Working standards of pharmaceutical grade Ondansetron Hydrochloride (Batch no. OS-1508) and Paracetamol (Batch no. 260738) were obtained as generous gifts from Suheka industries, Ahmedabad (Gujrat, India) and Bal Pharmaceuticals Ltd., Pune (Maharashtra, India) respectively. It was used without further purification and certified to contain 99.78 % and 99.30 % (w/w) on dry weight basis Ondansetron Hydrochloride and Paracetamol, respectively. Fixed dose combination surup (Ondem-P) containing 2 mg Ondansetron Hydrochloride and 125 mg

Paracetamol was purchased from Swami Samarth medical store, Pune, India. All chemicals and reagents used were of HPLC grade for HPLC & GR grade for HPTLC and were purchased from Merck Chemicals, India.

2.2 Instrumentation

For HPTLC, the samples were spotted in the form of bands 6 mm width with a Camag 100 microlitre sample syringe (Hamilton, Bonaduz, Switzerland) on silica gel precoated aluminum plate 60 $_{F\ 254}$, [(20 × 10 cm) with 250 µm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai] using a Camag Linomat V (Switzerland). Linear ascending development was carried out in 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25 ^{o}C ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 8 cm. Densitometric scanning was performed on Camag HPTLC scanner III in the reflectance-absorbance mode at 286 nm and operated by CATS software (V 3.15, Camag). The source of radiation utilized was deuterium lamp emitting continuous UV spectrum between 190 and 400 nm.

The HPLC system consisted of a Pump (model Jasco PU 2080), Intelligent LC pump with sampler programmed at 20 µl capacity per injection was used. The detector consisted of UV/ VIS (Jasco UV 2075) model operated at a wavelength of 286 nm. Data was integrated using Jasco Borwin version 1.5, LC-Net II/ADC system. The column used was PerfectSil-100 ODS-3–C_{18} (250 mm × 4.6 mm, 5.0 µ) from MZ-Analysetechnik GmbH, Germany.

2.3. Preparation of Standard Stock Solutions

Standard stock solutions of concentration 0.2 mg/ml of OND and 12.5 mg/ml of PARA were prepared separately using methanol. From the standard stock solution, the mixed standard solution was prepared using methanol to contain 0.04 mg/ml of OND and 2.5 mg/ml of PARA for HPTLC and 0.010 mg/ml of OND and 0.625 mg/ml of PARA for HPLC, respectively. The stock solution was stored at 2-8 °C, protected from light.

2.4 Optimization of HPLC and HPTLC Method

The HPLC and HPTLC procedure was optimized with a view to develop a simultaneous assay method for OND and PARA, respectively.

For HPTLC, the mixed standard stock solution (0.04 mg/ml of OND and 2.5 mg/ml of PARA) was spotted on to HPTLC plates and run in different solvent systems. Initially, toluene, methanol, acetone & formic acid were tried in different ratios. Toluene was used to impart the necessary non-polarity to mobile phase to obtain a suitable R_F value. Initially toluene, methanol, acetone & formic acid in the ratio of 5:2:2:0.01, v/v/v/v was

selected but R_F was found very close to each other (0.53 & 0.58 for OND & PARA, respectively). Then the formic acid was completely removed from mobile phase and the volume of toluene & acetone in mobile phase increased from 5 ml to 6 ml & 2 ml to 2.5 ml respectively to decrease R_F and improve separation. Finally, the mobile phase consisting of toluene: methanol: acetone in the ratio of 6:2:2.5, v/v/v was found optimum. In order to reduce the neckless effect HPTLC chamber was saturated for 20 min using saturation pads. The mobile phase was run up to a distance of 8 cm; which takes approximately 20 min for complete development of the HPTLC plate. The scanning wavelength selected was 286 nm where good response was observed for both OND and PARA. (Fig. 2)
For HPLC, the mixed standard stock solution (0.010 mg/ml of OND and 0.625 mg/ml of PARA) was injected. Diifferent ratios of acetonitrile and acetate buffer at different pH were tried but it was found that acetonitrile: ammonoum acetate buffer (50 mM) in the ratio 40:60 v/v, pH-6 adjusted with glacial acetic acid at flow rate 1 ml/min gives acceptable retention time (t_R), plates and good resolution for OND and PARA.

2.5 Validation of the method
Validation of the optimized HPTLC & HPLC method was carried out with respect to the following Parameters.
2.5.1 Linearity and range
From the mixed standard stock solution 0.04 mg/ml of OND and 2.5 mg/ml of PARA, 1 to 5 µl solutions were spotted on HPTLC plate to obtain final concentration of 40–200 ng/spot for OND and 2500-12500 ng/spot for PARA. Each concentration was applied in triplicate on the HPTLC plate.
For HPLC, the mixed standard stock solution (0.010 mg/ml of OND and 0.625 mg/ml of PARA) further diluted to get OND and PARA in the concentration range of 2-10 µg/ml and 125-625 µg/ml respectively. Linearity of the method was studied by injecting **five** concentrations of the drug prepared in the mobile phase in triplicate into the HPLC system keeping the injection volume constant.

2.5.2. Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations by HPTLC (40, 120, 200 ng/spot for OND and 2500, 7500, 12500 ng/spot for PARA) and HPLC (2, 6, 10 µg/ml for OND and 125, 375, 625 µg/ml for PARA) of the drug six times on the same day. The intermediate precision of the method was checked by repeating studies on three different days.

2.5.3 Limit of detection and limit of quantitaiton
LOD and LOQ were determined by measuring the magnitude of analytical background by spotting a blank and calculating the signal-to-noise ratio for OND and PARA by spotting a series of solutions until the signal-to-noise ratio of 3 for LOD and 10 for LOQ. To determine the LOD and LOQ, serial dilutions of mixed standard solution of OND and PARA was made from the standard stock solution. The samples were injected in LC system and applied to HPTLC plate and the chromatograms were run and measured signal from the samples was compared with those of blank samples.

2.5.4 Robustness of the method

Following the introduction of small changes in the mobile phase composition (± 0.1 ml for each component), the effects on the results were examined. Mobile phases having different compositions, e.g. toluene: methanol: acetone (6.1:2:2.5, v/v/v), (5.9:2:2.5, v/v/v), (6:2.1:2.5, v/v/v), (6:1.9:2.5, v/v/v) were tried and chromatograms were run. The amount of mobile phase was varied over the range of ± 5 %. The plates were prewashed with methanol and activated at 110°C for 2, 5, and 7 min respectively prior to chromatography. The time from spotting to chromatography and from chromatography to scanning was varied by ± 10 min.

To evaluate robustness of a HPLC method, few Parameters were deliberately varied. The Parameters included variation of flow rate, percentage of methanol in the mobile phase, pH of mobile phase. The resolution of drug in a mixture of stressed samples was studied by performing the analyses on a different different chromatographic system.

2.5.5 Specificity

The specificity of the method was determined by analyzing standard and drug and samples. The spot for OND and PARA in the samples was confirmed by comparing the R_F and spectrum of the spot with that of a standard. The peak purity of OND and PARA was determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M) and peak end (E).

2.5.6 Accuracy

Accuracy of the method was carried out by applying the method to drug sample (OND and PARA combination syrup) to which know amount of OND and PARA standard powder corresponding to 50, 100 and 150% of label claim had been added (Standard addition method), mixed and the powder was extracted and analyzed by running chromatogram in optimized mobile phase.

2.6. Analysis of a marketed formulation

To determine the content of OND of PARA in conventional syrup (Brand name: ONDEM-P, Label claim: 2 mg Ondansetron Hydrochloride and 125 mg Paracetamol per 5 ml of syrup), 1 ml of syrup equivalent to 0.4 mg OND and 25 mg PARA was transferred into a

10 ml volumetric flask containing 4 ml methanol, sonicated for 10 min and diluted to 10 ml with methanol. The resulting solution was centrifuged at 3000 r/min for 5 min and the drug content of the supernatant was determined (0.04 and 2.5 mg/ml for OND and PARA respectively). Then 4 ul of above filtered solution was applied to a HPTLC plate to produce a concentration of 160 and 10000 ng/spot for OND and PARA, respectively. 150 ul of the supernatant was diluted to 1 ml and 20 ul was injected in HPLC to produce a concentration of 6 ug/ml and 375 ug/ml for OND and PARA, respectively & developed in optimized mobile phase.
The analysis was repeated in triplicate. The possibility of excipient interference with the analysis was examined

3. Results and Discussion
The results of validation studies on simultaneous estimation method developed for OND and PARA in the current study involving toluene: methanol: acetone (6:2:2.5, v/v/v) and acetonitrile: ammonium acetate buffer (50 mM) [40:60] pH-6 adjusted with glacial acetic acid as the mobile phase for HPTLC and HPLC, respectively are given below.

3.1 Validation
3.1.1 Linearity
The drug response was linear (Table 1) over the concentration range between 40-200 ng/spot for OND and 2500-12500 ng/spot for PARA (Fig. 3) by HPTLC (r^2 = 0.9995 for OND and 0.9923 for PARA) and by HPLC over the concentration range between 2-10 ug/ml for OND and 125-625 ug/ml for PARA. (r^2= 0.9992 for OND and 0.9949 for PARA)

3.1.2. Precision
The results of the repeatability and intermediate precision experiments are shown in Table 2. The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were < 2 %, respectively as recommended by ICH guidelines.

3.1.3 LOD and LOQ
Signal-to-noise ratios of 3:1 and 10:1 were obtained for the LOD and LOQ respectively. By HPTLC, the LOD and LOQ were found to be 5.029 & 15.239 ng/spot for OND and 947.95 & 2872.58 ng/spot for PARA respectively. By HPLC, the LOD and LOQ were found to be 41.36 & 125.36 ng/spot for OND and 4846.56 & 14686.56 ng/spot for PARA respectively.
3.1.4 Robustness of the method

The standard deviation of the peak areas was calculated for each parameter and the RSD was found to be less than 2 %. The low values of the % RSD, as shown in Table 3 indicated robustness of the method by HPTLC. HPLC method also found robust as the RSD was less than 2 % although an experimental condition was slightly changed.

3.1.5 Specificity

The peak purity of OND and PARA was assessed by comparing their respective spectra at the peak start, apex and peak end positions of the spot i.e., r (S, M) = 0.9996 and r (M, E) = 0.9996. A good correlation (r = 0.9959) was also obtained between the standard and sample spectra of OND and PARA, respectively.

3.1.6 Recovery Studies

As shown from the data in Table 4 good recoveries of the OND and PARA in the range from 98.07 to 99.33 % by HPTLC and 98.05 to 99.11 % by HPLC were obtained at various added concentrations. The average recovery of three levels (nine determinations) for OND and PARA were 98.52 % (0.47) & 98.43 % (0.48) respectively by HPTLC and 98.75 % (0.25) & 98.58 % (0.53) respectively by HPLC.

3.2 Analysis of a formulation
Experimental results of the amount of OND and PARA in tablets, expressed as a percentage of label claims were in good agreement with the label claims thereby suggesting that there is no interference from any of the excipients which are normally present in tablets (Fig. 4 and 5). The drug content was found to be 96.78 % of OND and 96.25 % of PARA by HPTLC; 97.43 % of OND and 98.35 % of PARA by HPLC.

Conclusion
The developed HPLC and HPTLC technique is precise, specific and accurate. Statistical analysis proves that the method is suitable for the analysis of OND and PARA as bulk drug and in pharmaceutical formulation without any interference from the excipients. It may be extended to study the degradation kinetics of OND and PARA and also for its estimation in plasma and other biological fluids.

Acknowledgement
The authors would like to thank Suheka industries, Ahmedabad (Gujrat, India) and Bal Pharmaceuticals Ltd., Pune (Maharashtra, India) for providing a gift sample of standard Ondansetron Hydrochloride and Paracetamol. The authors would like to thank, Dr. K. R. Mahadik, Principal, Poona College of Pharmacy, Pune, India for providing necessary facilities to carry out the wok.

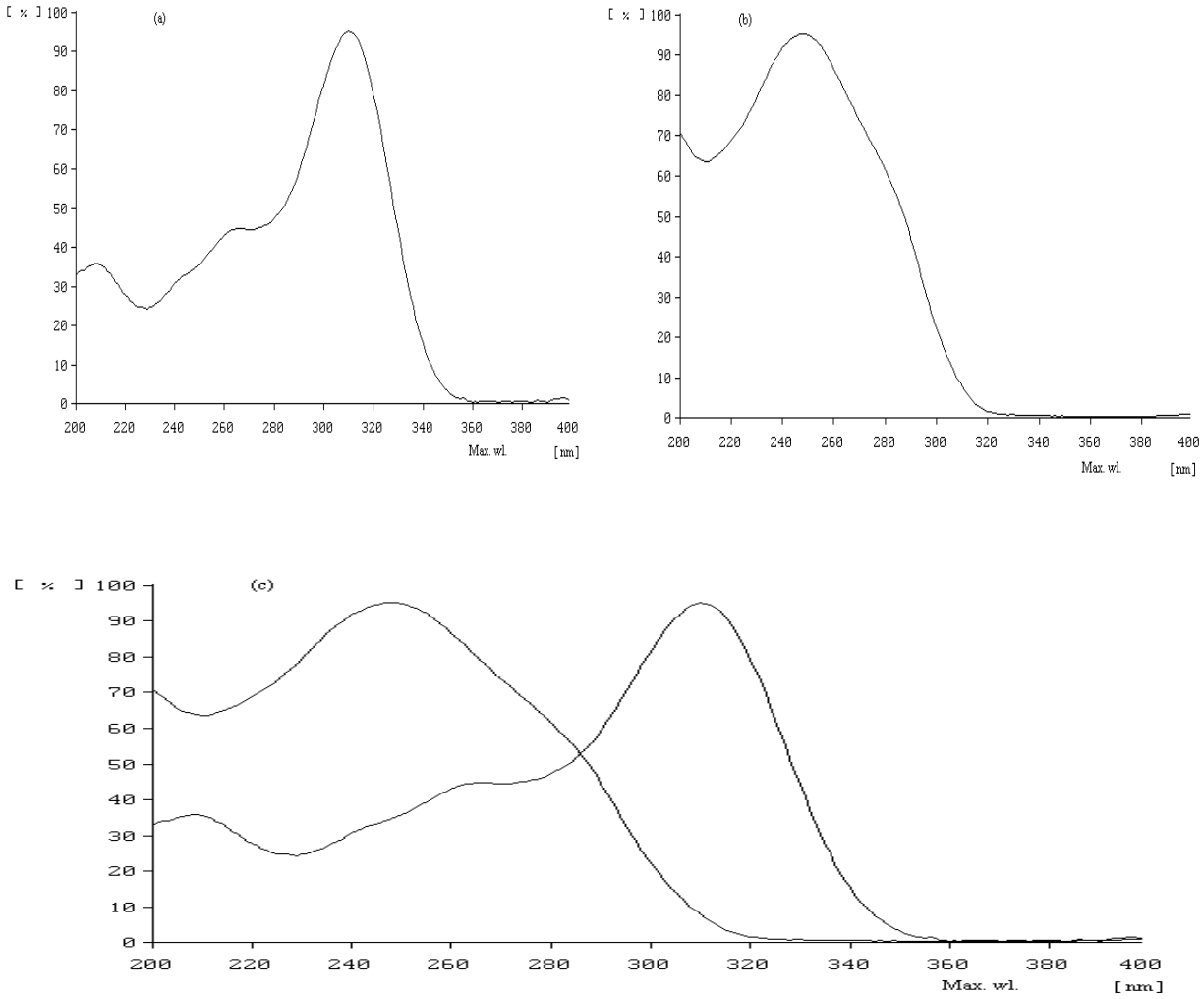

Fig 2 Representative spectrum of (a) OND, (b) PARA, (c) In-situ overlain spectrum of OND and PARA.

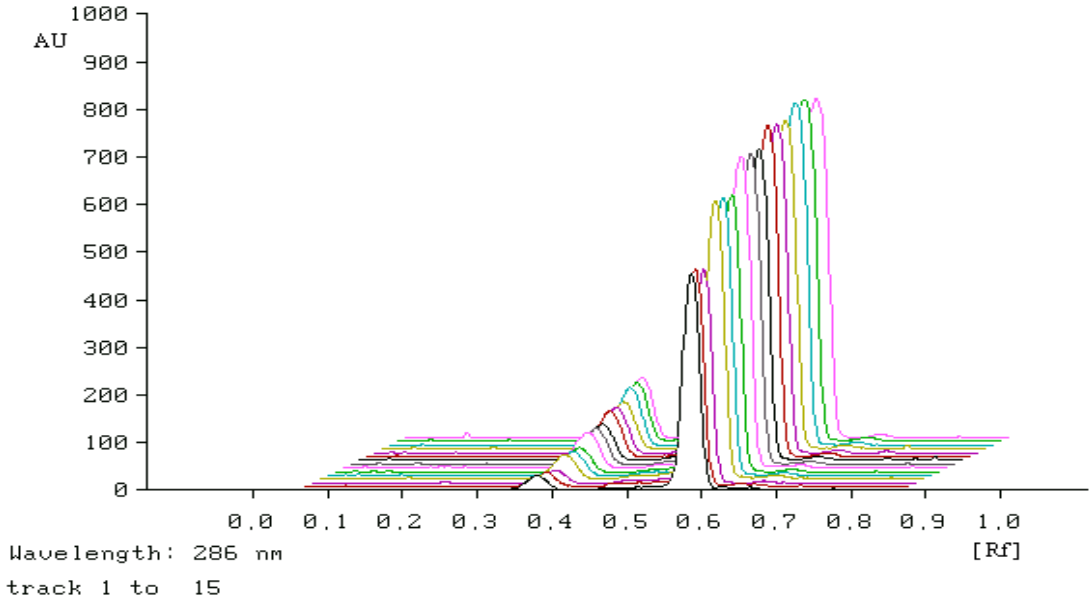

Fig. 3 Three-dimensional HPTLC chromatograms of calibration samples applied in triplicate on HPTLC plate.

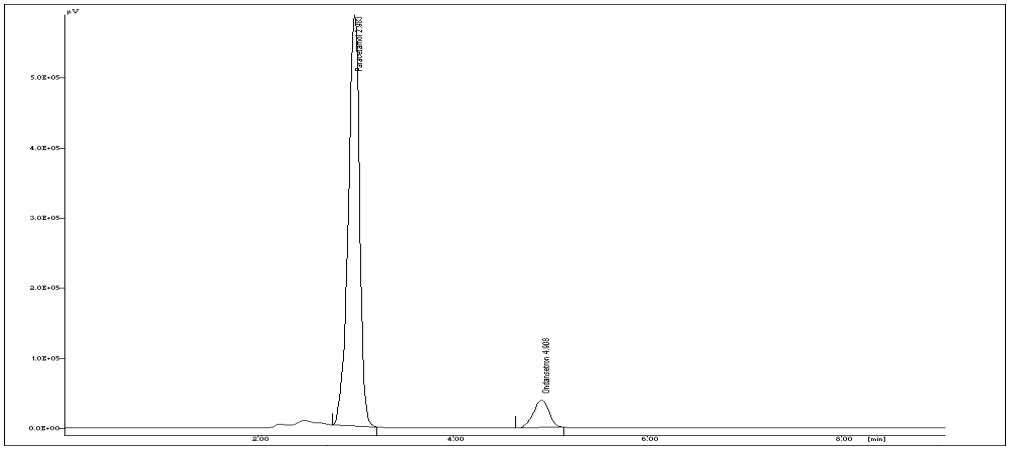

Fig. 4. Chromatogram of OND (t_R 4.8 min) and PARA (t_R 2.9 min) of formulation (ONDEM-P) showing no interference of excipients in analysis.

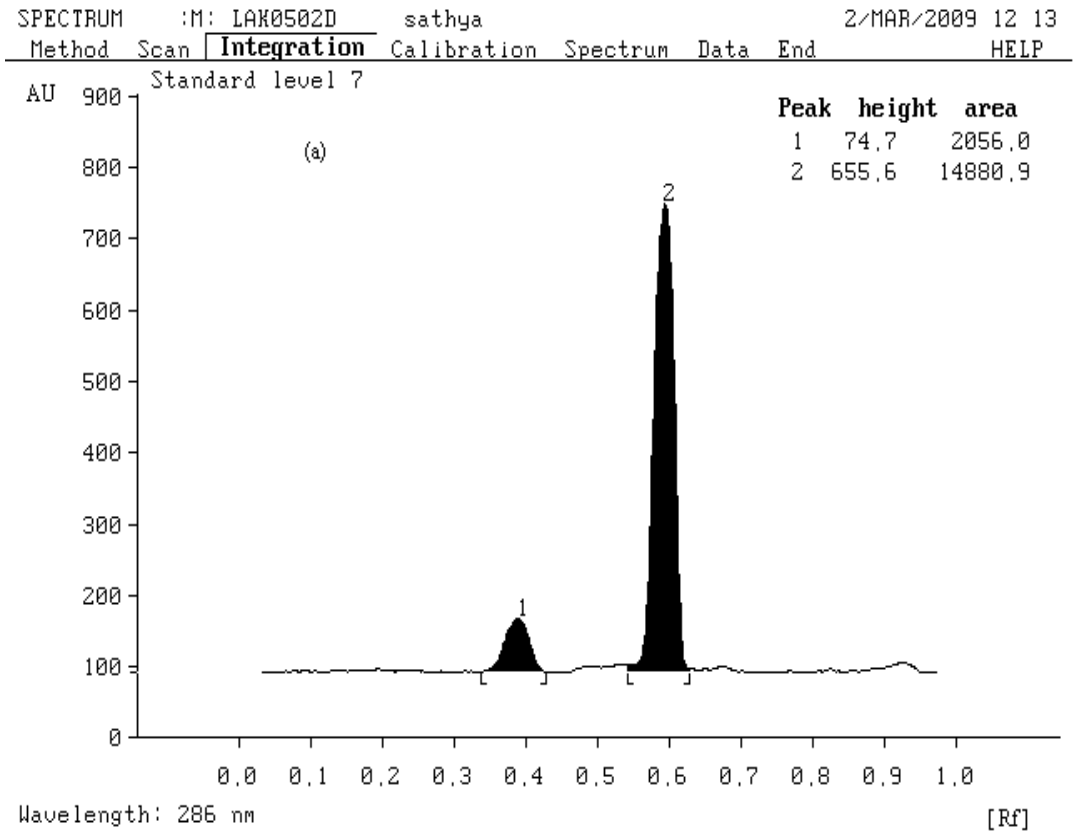

Fig. 5 Densitogram of OND (R_F 0.39) and PARA (R_F 0.59) of formulation (ONDEM-P) showing no interference of excipients in analysis.

Table 1. Linear regression data for calibration curves.

(a) **By HPLC :**

Parameters	**OND**	**PARA**
Linearity range	2-10 ug/ml	125-625 ug/ml
r^2	0.9992	0.9949
Slope	20536	13668
Intercept	1961.6	178894

(b) By HPTLC :

Parameters	**OND**	**PARA**
Linearity range	40-200 ng/spot	2500-2500 ng/spot
r^2	0.9995	0.9923
Slope	15348	1099.3
Intercept	153.34	6248.4

Table 2. Precision studies

a) By HPLC

Concentration (ug/ml)	**Repeatability (*n= 6*)**		**Intermediate precision (*n= 6*)**	
	Measured concentration ± SD	**RSD (%)**	**Measured concentration ± SD**	**RSD (%)**
OND				
2	1.94± 0.0	1.817	1.96± 0.0	1.442
6	5.87± 0.0	1.927	5.84± 0.0	1.5721
10	9.63± 0.0	1.909	9.66± 0.1	1.097
PARA				
125	122.32 ± 1.8	1.549	122.5 ± 2.2	1.870
375	370.25 ± 4.8	1.300	371.14 ± 3.8	1.026
625	613.94 ± 7.4	1.217	615.29 ±7.1	1.159

By HPTLC

Concentration (ng/spot)	**Repeatability (*n= 6*)**		**Intermediate precision (*n= 6*)**	
	Measured concentration ± SD	**RSD (%)**	**Measured concentration ± SD**	**RSD (%)**
OND				
40	38.8± 0.3	0.827	39.7± 0.5	1.262
120	119.2± 0.5	0.438	118.8 ± 0.9	0.833
200	197.3± 2.6	1.365	196.6 ± 2	1.035
PARA				
2500	2450.4 ± 2.6	0.106	122.5 ± 2.2	0.103
7500	7398.8 ± 3	0.041	371.14 ± 3.8	0.022
12500	12237.3 ± 2.8	0.023	615.29 ±7.1	0.015

Table 3. Robustness testing (n = 3)

Parameter area	**% RSD**	**SD of peak area for OND**	**% RSD**	**SD of peak for PARA**
Mobile phase composition (± 0.1 mL)	2.15	1.23	2.76	1.08
Amount of mobile phase (± 5%)	4.12	1.45	1.33	1.84
Time from spotting to (± 10 min.)	3.29	1.31	1.15	2.63
Time from chromatography to scanning (± 10 min.)	2.76	1.82	1.52	3.63

Table 4. Recovery studies

a) By HPLC

Drug Recovery (%)	**Label claim (mg per ml)**	**Amount added (%)**	**Total amount (mg)**	**Amount recovered (mg) ± SD**	**%RSD**
OND					
98.50	0.4	50	0.6	0.59± 0.0	1.435
98.75	0.4	100	0.8	0.79± 0.0	1.431
99	0.4	150	1	0.99± 0.0	1.070
PARA					
98.59	25	50	37.5	36.97 ±1.2	1.009
98.05	25	100	50	49.02 ± 4.5	1.866
99.11	25	150	62.5	61.94 ±3.4	0.932

a) By HPTLC :

Drug Recovery (%)	**Label claim (mg per ml)**	**Amount added (%)**	**Total amount (mg)**	**Amount recovered (mg) ± SD**	**%RSD**
OND					
98.07	0.4	50	0.6	0.588 ± 0.8	1.405
98.47	0.4	100	0.8	0.787 ± 1	0.855
99.02	0.4	150	1	0.99 ± 0.9	0.527
PARA					
99.33	25	50	37.5	37.24 ±6.6	0.178
98.79	25	100	50	49.39 ±8.6	0.116
98.37	25	150	62.5	61.48 ±11.67	0.105

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