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Short communication

Development of characterization methods for entacapone in a pharmaceutical bulk

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ABSTRACT

A comprehensive approach was taken to develop analytical procedures for the characterization of entacapone in a pharmaceutical bulk. A novel reversed-phase HPLC method was developed and validated for the assay of entacapone and the determination of impurities. The method employed a C18 column, a mobile phase of potassium phosphate buffer (pH 2.75, 30 mM)–methanol (50:50, v/v) at a flow rate of 1.0 mL/min, and ultraviolet (UV) detection at 310 nm. The method was linear over the range from 50% to 150% of the assay concentration (0.2 mg/mL). The limit of quantitation was 0.13 μg/mL, and the limit of detection was 0.05 μg/mL. Average recovery was 100.10% with a relative standard deviation ($N = 9$) of 0.45%. Degradation studies showed that entacapone eluted as a spectrally pure peak and was well resolved from its degradation products. The method was specific, sensitive, precise, linear, and accurate.

UV and infrared spectroscopy (IR) were suitable as identification tests. The UV and FTIR spectra were acquired from a candidate reference standard and two commercial bulks. All materials exhibited maxima and minima at the same wavelengths. Water sorption analysis showed that entacapone was not affected by atmospheric moisture.

These methods can be used for qualitative and quantitative analysis of entacapone in a pharmaceutical bulk.

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1. Introduction

The US Pharmacopeial Convention (USP) establishes publicly recognized standards to ensure the quality and safety of medicines. USP strives to keep its monographs current with advances in technology and the growing number of new drugs on the market each year. This study was conducted to support the development of an entacapone monograph.

Entacapone [(*E*)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)-*N*,*N*-diethylprop-2-enamide] is a second-generation catechol-*O*-methyltransferase (COMT) inhibitor for adjunctive treatment with levodopa in Parkinson disease [1]. A few methods have been reported for the quantification of entacapone and its metabolites in biological fluids [2–5].

Recently, a few analytical methods for the determination of entacapone were published and most of them reported high-performance liquid chromatography (HPLC) assay of entacapone in dosage forms [6–9], but none of these show a stability-indicating method. At present only one paper refers to a pharmaceutical bulk and reports the stress testing. Shetty et al. [10] developed an HPLC method for entacapone assay in pharmaceutical bulks. However, the method used gradient elution and required a long run time, and the stress study was performed on solid samples. No results showed the separation between *E*-entacapone and its isomer *Z*-entacapone, which is the main impurity in the synthesis of entacapone. This paper describes the simple, fast, reliable method for determination of entacapone in a pharmaceutical bulk. A reversed-phase isocratic HPLC method was developed. An accelerated stress degradation study was performed, and results showed that the degradants and impurities were well resolved from the main peak, confirming that the method is stability indicating. The system suitability test was developed as a part of a validation package for pharmacopeial methods.

Because pharmacopeial monographs focus on chemical identification and the assessment of quality and purity, the aim of this study was to develop analytical methods for characterization and quantitation of entacapone in a pharmaceutical bulk. The primary focus was on HPLC method development for assay and chromatographic purity, but identification and moisture adsorption were also investigated.

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2. Experimental

2.1. Materials

Entacapone candidate and chloramphenicol reference standards were obtained from USP (Rockville, MD, USA). Two entacapone bulk materials were purchased from American Custom Chemical Corporation (San Diego, CA, USA) and LGM Pharmaceutical Incorporation (Boca Raton, FL, USA).

HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Potassium phosphate monobasic, hydrochloric acid, and 30% hydrogen peroxide were also obtained from Fisher Scientific. Potassium phosphate dibasic and phosphoric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Sodium hydroxide was obtained from Sigma (St. Louis, MO, USA), and potassium bromide was purchased from McCarthy Scientific Co. (Fallbrook, CA, USA). All chemicals were of analytical grade.

Water was obtained from a Milli-Q water purification system (Millipore, Billerica, MA, USA).

2.2. Instrumental and analytical conditions

2.2.1. Chromatographic system

The primary HPLC system was an Agilent 1200 series HPLC system containing a quaternary pump, vacuum degasser, thermostated column compartment, autosampler, and a diode-array UV–vis detector with ChemStation Software version B.01.01 (Wilmington, DE, USA). The second HPLC system was a Shimadzu LC-20A series HPLC system with VP-7 software (Columbia, MD, USA).

2.2.1.1. Chromatographic conditions. Chromatographic separations were performed on Symmetry C_{18} 5 μm, 4.6 mm × 250 mm column (Waters, Milford, MA, USA) and a Prodigy ODS-3, 5-μm, 4.6 mm × 250 mm column (Phenomenex, Torrance, CA, USA). Both systems used UV detection at 310 nm, a 20-μL injection volume, and a 1-mL/min flow rate. The mobile phase consisted of methanol–potassium phosphate buffer (pH 2.75, 30 mM) (50:50, v/v).

2.2.1.2. Preparation of solutions. The buffer was prepared by dissolving 2.7 g of monobasic potassium phosphate and 1.7 g of dibasic potassium phosphate in 1 L of water and adjusting the pH to 2.75 with phosphoric acid.

Mobile phase was prepared by mixing equal volumes of buffer and methanol.

A mixture of the prepared buffer and methanol (2:1, v/v) was used as a diluent for sample preparation.

2.2.1.3. Standard and sample preparation. Entacapone is practically insoluble in water. Therefore, the stock solution was prepared in methanol. For standard and assay preparations entacapone was initially dissolved in methanol and then diluted with diluent.

An entacapone standard solution containing 0.2 mg/mL was prepared in a 25-mL volumetric flask by dissolving 5 mg of entacapone candidate reference standard (RS) in 5 mL of methanol and diluting to volume with diluent. The assay solutions were prepared the same way from two commercial bulks. The solutions were sonicated to ensure that the samples dissolved.

A system suitability solution was prepared by exposing a standard solution to UV and visible light for 3–5 h.

A resolution solution containing 0.4 mg/mL of chloramphenicol and 0.2 mg/mL of entacapone was prepared in a 25-mL volumetric flask by dissolving a 10-mg portion of chloramphenicol and 5 mg of entacapone in 5 mL of methanol and then diluting to volume with diluent. The solution was sonicated to ensure that the sample dissolved.

2.2.1.4. Method validation. Stock solution of entacapone at 5 mg/mL was prepared in methanol.

Linearity solutions were prepared at 75%, 100%, 125%, and 150% levels from the assay-level concentration (0.2 mg/mL) by serial dilutions of the stock solution with diluent.

Limit of detection (LOD) and limit of quantitation (LOQ) solutions were prepared by serial dilutions of the stock solution with diluent.

For degradation studies acid-, base-, and oxidizer-stressed samples were prepared by mixing 1.0 mL of entacapone stock solution with 1.0 mL of 1 M hydrochloric acid, 1 M sodium hydroxide, or 3% hydrogen peroxide and maintaining the samples protected from light at ambient temperature for 22 h (2 h for base-stressed sample). Acid- and base-treated samples were neutralized with 1.0 mL of 1 M sodium hydroxide and 1 M hydrochloric acid, respectively. Then, all samples were diluted with diluent to a final concentration of 0.2 mg/mL. For thermal degradation, a portion of solid entacapone was dried at 60 °C for 24 h, and a 0.2 mg/mL solution was prepared from the dried material. In addition, the solution was prepared from sample used in the water sorption experiment.

Light-stressed samples were prepared by exposing a standard solution to UV and visible light for different periods of time.

For accuracy studies entacapone solutions were prepared at three levels corresponding to 80%, 100%, and 120% of the assay level. For each level three sets with different weighing were prepared and were injected in triplicate.

For intermediate precision entacapone solutions were prepared at the 100% level from each bulk and were analyzed against standard solution. Five individual preparations were made for each bulk and reference standard material, and two replicate injections were made for each preparation. The experiment was conducted by two chemists, on two separate days, on different chromatographic systems.

All entacapone solutions were prepared in low actinic glass and were protected from light.

2.2.2. Water sorption

Water adsorption and desorption data were obtained using an automatic gravimetric water vapor adsorption analyzer (SGA-100, VTI Corporation, Hialeah, FL, USA). Nominal 25-mg samples were exposed to relative humidity (RH) from 10% to 90% to 10% RH in 10% steps at 25 °C, dried in the instrument at 105 °C for 3 h, cooled to 25 °C, and then exposed to 10% to 90% to 10% RH in 10% steps at 25 °C.

2.2.3. UV–vis spectroscopy

UV–vis absorption spectra were obtained on a PerkinElmer Lambda 35 UV–vis double-beam spectrophotometer (Waltham, MA, USA), equipped with UV-Win Lab version 2.85 software for data processing. Measurements were done at room temperature in the 203–700 nm spectral range. Entacapone UV solutions were prepared at 14 μg/mL in hydrochloric acid (1 M) and methanol (1:10, v/v). Data were obtained in triplicate.

2.2.4. FTIR spectroscopy

FTIR spectra were recorded as KBr (potassium bromide) dispersions (1:150, w/w) on a Nicolet Nexus-870 FTIR spectrophotometer (Thermo Scientific, Madison, WI, USA) with Omnic version 5.1 software. Spectra were collected from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}, 32 scans/spectrum.

3. Results and discussion

3.1. Chromatographic analysis

3.1.1. Method development

Chromatographic conditions were optimized in order to develop a stability-indicating assay method. Entacapone exists in two stereo-isomeric forms: the major *E*-isomer and minor *Z*-isomer. Entacapone solution was exposed to UV light to form an isomer mixture that was used as a resolution solution during method development [4]. Two organic solvents (acetonitrile and methanol) were tested for mobile phase composition on a Waters Symmetry C_{18} column. With a mobile phase of acetonitrile–phosphate buffer (pH 2.75, 30 mM) (40:60, v/v) the *Z*-entacapone and *E*-entacapone peaks eluted at 8.2 and 8.9 min, respectively, with a resolution of 2.3. Separation was improved by changing the organic solvent in the mobile phase to methanol. With a mobile phase of methanol–buffer (50:50, v/v) at a flow rate of 1.0 mL/min, the entacapone peaks eluted at 9.4 min (*Z*-isomer) and 14.2 min (*E*-isomer), the resolution between the *Z*- and *E*-isomers was 10, and additional peaks were detected. Therefore, the mobile phase methanol–buffer, wavelength of 310 nm, and working standard concentration of entacapone at 0.2 mg/mL were chosen for the final work.

In light-exposed solutions of entacapone the stable *Z*-isomer was formed. The percent total detected area (% TDA) of *Z*-isomers increased from 0.05% in a fresh solution of entacapone to 5.9% TDA and 17.2% TDA in a 5-h and 22-h light-exposed solution, respectively. The % TDA of entacapone decreased from 99.8% to 93.8% and 81.9%, respectively, for the same time periods. Solutions of entacapone exposed to light for 3–5 h were considered appropriate for use as a system suitability test.

For use as internal standard, additional resolution probes were also investigated. Two structurally related compounds—tolcapone and chloramphenicol—were considered. Because the tolcapone peak eluted very late (more than 60 min), chloramphenicol, which eluted at 7.3 min, was chosen as a possible internal standard or additional resolution probe: It was stable in solution, and its chromatographic behavior was similar to that of entacapone under different chromatographic conditions. As a result, both the light-exposed system suitability solution and the chloramphenicol-containing resolution solution were used as resolution probes in further work.

3.1.2. System suitability

According to USP, system suitability testing is an integral part of analytical methods [11]. A system suitability test was developed to evaluate chromatographic parameters of the system. Replicate injections of the system suitability solution and resolution solution were made throughout the validation process. Resolution between chloramphenicol and *E*-entacapone was always greater than 13, and that between the *E* and *Z* isomers was greater than 9 (Fig. 1). Tailing for the three peaks was between 1.0 and 1.4, efficiencies for the three peaks were greater than 8500 theoretical plates, and the precision for the entacapone peak for five replicate injections was less than 1.0%. The relative retention times were 0.5, 0.7, and 1.0 for chloramphenicol, *Z*-entacapone, and *E*-entacapone, respectively.

3.1.3. Validation of the HPLC method

3.1.3.1. Specificity. USP defines specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components [11]. The specificity of the HPLC method was examined by peak identification and stress studies. No interferences appeared at the retention time of entacapone in the chromatograms from the blank (diluent) and chloramphenicol solutions. Possible interferences from the degradants were determined by comparing the chromatograms of solvent blanks and unstressed and stressed samples of the entacapone standard solution. Several additional degradation peaks were observed, and some existing impurities increased in the stressed samples. Degradation was observed for all stressors except heat and humidity exposure. The greatest degradation took place under basic conditions. All additional peaks were well separated from the parent peak with resolutions greater than 2 (Fig. 2). Diode-array detection was employed for spectral purity analysis of the entacapone peak in the stressed samples over a range of 200–700 nm. The entacapone peak was spectrally pure in all stress samples (peak purity value obtained by using ChemStation software was 0.999).

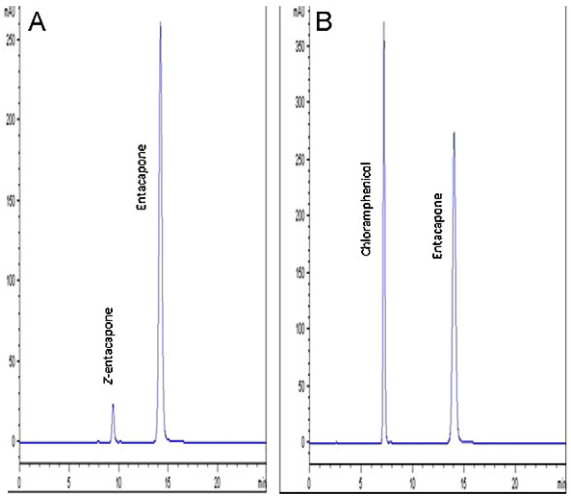

Fig. 1. Typical HPLC chromatograms of (A) system suitability solution (standard solution of entacapone, 0.2 mg/mL) exposed to light for 4 h; (B) resolution solution consisting of a mixture of entacapone (0.2 mg/mL) and chloramphenicol (0.4 mg/mL). Approximate relative retention times: chloramphenicol, 0.5; *Z*-entacapone, 0.7; entacapone 1.0.

3.1.3.2. Linearity. According to USP linearity is the ability within range to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample [11]. The linearity of the method for the entacapone peak area response was examined at five points on a calibration curve in concentration ranges from 0.1 to 0.3 mg/mL. Linearity experiments were conducted on three consecutive days with different stock solution preparations. The linear regression equation was $y = 30046x + 64$. The correlation coefficient, *r*, was >0.999, and the *y*-intercept was less than 1.0% when compared to the response at the 100% level in every experiment.

3.1.3.3. Accuracy and precision. Instrument precision was determined by replicate injections of the resolution solutions. The second chemist also performed the same analysis. The relative standard deviation (%RSD) for the entacapone peak response was below 1.0% for all components.

USP precision is defined as the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample [11], and accuracy is the closeness of test results to the true value.

The intra-day precision and accuracy of the proposed method were determined at three different levels corresponding to 80%, 100%, and 120% (w/w) of the nominal analytical concentration of 0.2 mg/mL. The samples were analyzed using a standard curve calibration performed the same day. The mean recovery data obtained for each level as well as for all levels combined were within ±2% (Table 1). The %RSD for the relative response factor (RF) for all preparations ($N = 9$) was less than 1.0%.

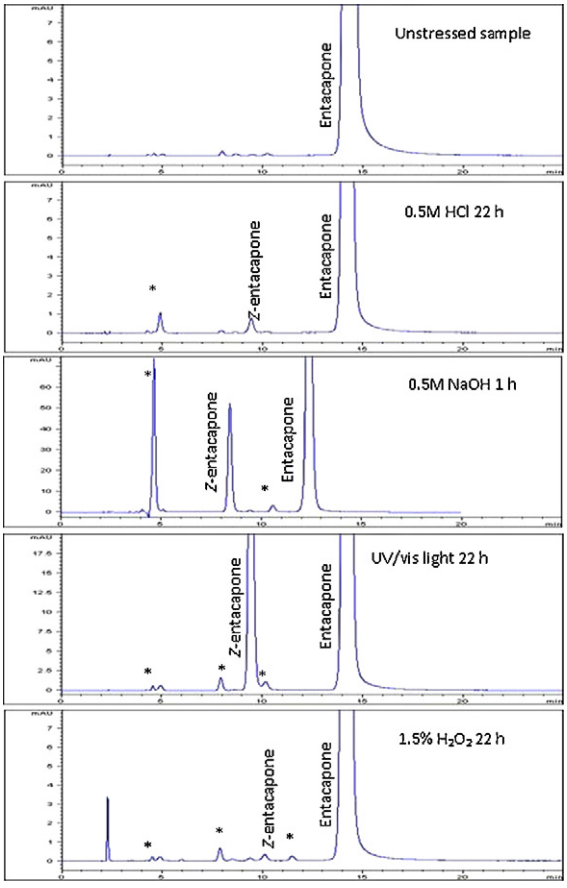

Fig. 2. Typical chromatograms recorded during forced degradation studies. All unknown and degradant peaks, including *Z*-entacapone, were resolved from the entacapone peak with resolutions >2.0.

Intermediate precision was determined by the assay of the two commercial bulks vs the candidate RS. The assay results for bulk 1 were 100.3% and 100.0% for chemist 1 and chemist 2, respectively, and those for bulk 2 were 99.2% and 99.6% for chemists 1 and 2, respectively. The intermediate precision, %RSD, was less than 1.0% for each analyzed sample, and the absolute difference between the two chemists was less than 1.0%.

The consistent and low %RSD values obtained for all experiments indicated that this method was highly precise.

3.1.3.4. LOD and LOQ. LOD and LOQ were determined based on signal-to-noise ratios by comparing measured signals from samples with known low concentrations of entacapone with the signal from the baseline. The LOD at three times peak-to-peak noise was found to be 0.05 μg/mL or 0.03% of the assay concentration. The LOQ at 10 times peak-to peak noise was 0.13 μg/mL (0.07% of the assay concentration), and the %RSD from six replicate injections of an LOQ solution was 2.7%.

3.1.3.5. Robustness. Robustness was determined by varying the separation parameters: mobile phase composition (±2%), buffer pH (±0.5 pH unit), column temperature (±5 °C), flow rate (±0.2 mL/min), and UV wavelength (±5 nm). The resolution probe solutions were injected and the area responses or ratio of area responses, column efficiency, retention times, resolutions between *Z*- and *E*-isomers and chloramphenicol and entacapone, the tailing factor, and the impurity profile were compared with the proposed method. The response of entacapone significantly changed with the pH of the mobile phase. Entacapone area decreased by 41% at pH 3.25 and increased by 15% at pH 2.25. There was no effect on the chloramphenicol response. At the same time, the peak area of chloramphenicol significantly changed with variations in detection wavelength. Other chromatographic parameters (tailing, resolutions, column efficiency, and relative retention time) were not affected. The method was found to be robust under the experimental conditions studied.

3.1.3.6. Ruggedness. The ruggedness of the method was determined by analyzing the candidate RS and two commercial bulks by two different analysts using two different columns on different days and two different instruments using the proposed method. The results showed that method was sufficiently rugged with respect to all critical parameters (Table 2).

3.1.3.7. Stability of analytical solutions. Stability was monitored by analyzing a *standard solution* aged at room temperature while protected from light and comparing the chromatographic profile to that of freshly prepared standards. After 16 h, the entacapone peak area decreased by 0.3% and after 5 days by 7%.

3.1.4. Assay and chromatographic purity

The proposed method was applied for assay of commercial bulks (see Section 3.1.3.3). The assay concentration of 0.2 mg/mL was acceptable for use in a chromatographic purity test. A few impurities were detected, and each was less than 0.1% total detected area (TDA) for the candidate RS and two bulks. Total additional peaks were less than 0.3% TDA.

3.2. Moisture adsorption/desorption study

Chemical stability and physical properties of solid drugs/APIs can be affected by moisture. Water sorption analysis was performed

Table 1
Accuracy and precision results for entacapone at different concentrations.

Assay % level	Concentration known (mg/mL)	RF[a]	Concentration found[b] (mg/mL)	% recovery[c] (average ± SD)
80	0.161	29,865	0.161	99.84 ± 0.30
	0.158	29,900	0.158	
	0.155	29,722	0.154	
100	0.198	29,909	0.198	99.97 ± 0.27
	0.203	29,830	0.203	
	0.200	29,746	0.199	
120	0.240	30,108	0.243	100.49 ± 0.49
	0.249	29,957	0.252	
	0.240	29,800	0.240	
Average (*N* = 9)		29,871	Average (*N* = 9)	100.10
%RSD		0.39	%RSD	0.45

[a] RF (response factor), area response/known concentration.
[b] Concentration found = (area − intercept)/slope (based on linearity curve determined the same day: $y = 29682.34x + 30.88$).
[c] % recovery = 100 × concentration found/concentration known.

Table 2
Results for ruggedness experiment (comparison of two analysts/systems).

Chromatographic parameters	Agilent HPLC, Waters Symmetry column Analyst/system 1			Shimadzu HPLC, Phenomenex Prodigy column Analyst/system 2		
	Chloramphenicol	*Z*-entacapone	Entacapone	Chloramphenicol	*Z*-entacapone	Entacapone
Retention time (min)	6.6	8.4	12.3	7.2	9.3	13.7
Tailing	1.1	1.0	1.1	1.4	1.2	1.3
Efficiency[a]	9511	9451	10,016	10,258	10,664	12134
Resolution	14.9[b]	9.3[c]	N/A	16.7[b]	10.1[c]	N/A
Precision (%RSD)[d]	0.31	N/A	0.15	0.32	N/A	0.04

	Entacapone RS	Bulk 1	Bulk 2	Entacapone RS	Bulk 1	Bulk 2
Assay value[e] (%)	N/A	100.3 ± 0.6	99.2 ± 0.6	N/A	100.0 ± 0.4	99.6 ± 0.7
Chromatographic purity						
% TDA (entacapone peak)	99.86	99.83	99.72	99.85	99.86	99.75
Number of additional peaks	7	6	7	8	7	7
SUM of additional peaks (% TDA)	0.14	0.17	0.28	0.15	0.14	0.25

[a] Theoretical plates/column.
[b] Between chloramphenicol and entacapone.
[c] Between *Z*-entacapone and entacapone.
[d] Replicate injections.
[e] Determined from five preparations.

to examine the effect of humidity on entacapone. Entacapone was unaffected by atmospheric moisture: The weight change between 10% and 90% RH was less than 0.06%. Based on this result, a loss-on-drying test is recommended to remove possible residual solvents.

3.3. Spectrophotometric tests

USP General Chapter *Spectrophotometric Identification Tests* ⟨197⟩ sis based on profile comparison in which the spectrum of the substance is compared with the spectrum of the RS [12]. UV and IR spectroscopy were examined as identification tests.

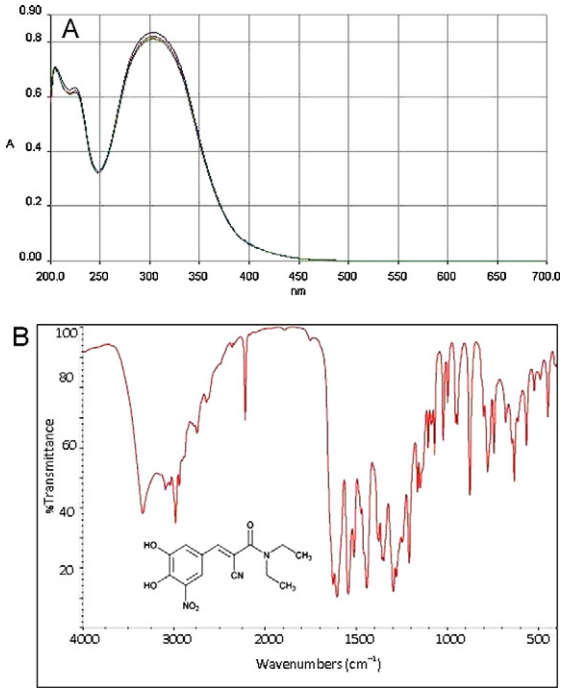

Fig. 3. Identification tests for entacapone in pharmaceutical bulk: (A) overlay of UV spectra for candidate RS and commercial bulks; (B) typical FTIR spectrum.

3.3.1. UV/vis absorption

UV spectra were measured on the candidate RS and two bulk materials. The spectra were in good agreement, exhibiting maxima and minima of similar intensities at the same wavelengths. The maximum absorbances were at 224 and 303 nm, and absorptivities at these wavelengths did not differ by more than 1%.

3.3.2. FTIR

Two chemists obtained IR absorption spectra (400–4000 cm^{-1}) for the entacapone candidate RS and the two commercial bulks. No differences were observed in the spectra obtained on different days or by different analysts. The spectra were in good agreement with each other and with a published spectrum [13], exhibiting maxima and minima at the same wavelengths between 650 cm^{-1} and 2000 cm^{-1}. In the IR spectrum, the sharp absorption band at 2217 cm^{-1} due to cyano-group stretching, the absorption bands at 3000 cm^{-1} due to C–H stretching, and those at 3340 cm^{-1} due to the phenol O–H stretching are consistent with structure.

Based on our data, both UV and FTIR methods can be used as identification test for entacapone (Fig. 3).

4. Conclusion

The methods developed provide independent and appropriate procedures for the characterization of entacapone. A simple, fast isocratic HPLC method for the assay and chromatographic purity test for entacapone in pharmaceutical bulk was developed. The proposed HPLC method successfully demonstrates chromatographic separation of entacapone from organic impurities and possible degradation products. The method was accurate, precise, reproducible, specific, and linear. FTIR and UV spectroscopy were suitable for identification tests. These methods can be employed in the analysis of the quality of entacapone in pharmaceutical bulks.

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