Original Article

Stability-indicating methods for the determination of racecadotril in the presence of its degradation products

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Summary **Three stability-indicating methods were developed for the determination of racecadotril (RCT) in the presence of its alkaline degradation products. The first was an high-pressure liquid chromatography (HPLC) method in which efficient chromatographic separation was achieved on a C_{18} analytical column and a mobile phase of acetonitrile-methanol-water-acetic acid (52:28:20:0.1, v/v/v/v). Linearity was obtained in the range of 4-40 µg/mL with mean accuracy of 99.5 ± 0.88%. The second method was a densitometric evaluation of thin-layer chromatograms of the drug using a mobile phase of isopropanol-ammonia (33%)-*n*-hexane (9:0.5:20, v/v/v). The chromatograms were scanned at 232 nm, a wavelength at which RCT can be readily separated from its degradation products and determined in the range of 2-20 µg per spot with mean accuracy of 99.5 ± 0.56%. The third method is based on the use of first-derivative spectrophotometry (D_1) at 240 nm, and the drug was determined in the range of 5-40 µg/mL with mean accuracy of 99.2 ± 1.02%. The three methods provided satisfactory recovery of the intact drug (100.8 ± 0.82, 100.4 ± 0.55, and 99.9 ± 0.72%, respectively) in the presence of up to 90% of its degradation products. Determination was also successful when analyzing RCT in a formulation in the form of acetorphan packets. Results were statistically analyzed and found to be in accordance with those given by a reported method.**

Keywords: Stability-indicating methods, degradation, racecadotril, quality control

1. Introduction

Racecadotril (RCT), *N*-[(R,S)-3-acetylmercapto-2-benzyl propanoyl] glycine benzyl ester, is a new anti-diarrheal pro-drug (*1*). In peripheral tissue membranes, RCT is converted into thiorphan, which inhibits the enzyme enkephalinase. As a result, enkephalin concentration increases, leading to activation of opioid receptors and a decrease in the cyclic adenosine monophosphate level. This in turn results in reduced secretion of water and electrolytes into the intestinal lumen (*2*,*3*).

A survey of the literature revealed few analytical methods for the determination of RCT, including spectrophotometric methods (*4*) and high-pressure liquid chromatography (HPLC) (*5*-*8*). In the present work, three simple, selective, and validated methods of HPLC, densitometry and first derivative (D_1) spectrophotometry were developed to quantify a drug in its pure form, in a pharmaceutical formulation, and in mixtures with its degradation products.

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2. Materials and Methods

2.1. *Reagents*

Pure RCT was purchased from Egyptian Pharmaceutical and Chemical Industry (EPCI), Cairo, Egypt and had a purity of 99.98% according to the supplier. Acetorphan packets (B.N.050; EPCI) containing 30 mg RCT per packet were purchased from a local market. All other reagents were analytical grade.

2.2. *Standard solutions*

A 2 mg/mL methanolic solution of pure RCT was prepared for use with the densitometric method. Further dilution was done to provide a methanolic solution of 0.1 mg/mL RCT for use with the HPLC and derivative methods.

2.3. *Degraded solutions*

About 100 mg of RCT were accurately weighed and transferred to a 100 mL round flask. Fifty mL of 0.1 N NaOH were added and heated under reflux for 2 h. After cooling, the pH was adjusted to 7 using 0.5 N HCl and mixture was then evaporated under a vacuum to dryness and extracted twice with 20 mL of methanol. The result was filtered into 50-mL volumetric flask and completed to volume with methanol to obtain an alkali-induced degradation solution containing degradation products derived from 2 mg/mL RCT for use with the densitometric method. Dilution was carried out by transferring 2.5 mL of each solution to a separate 50-mL volumetric flask and volume was completed with methanol to provide a solution labeled to contain degradation products equivalent to 0.1 mg/mL RCT.

2.4. *Linearity*

2.4.1. *HPLC method*

The HPLC instrument (AGLIENT 1500, USA) used consisted of an Agilent pump, equipped with a variable wavelength detector and a 20 µL volume injection loop, and an Eclipse C_{18} RP-column (1.8 µm, 50 × 4.6 mm *i.d.*).

Aliquots from the methanolic drug solution (0.1 mg/mL) equivalent to 0.04-0.4 mg RCT were transferred to a series of 10-mL volumetric flasks and diluted to volume with methanol. Twenty µL injections from each solution were chromatographed on the Eclipse C_{18} RP-column using a mobile phase of acetonitrile-methanol-water-acetic acid (52:28:20:0.1, v/v/v/v) at a flow rate of 0.7 mL/min and UV detection at 232 nm. The calculated peak areas were plotted with respect to the drug concentration and the regression parameters were deduced.

2.4.2. *Densitometric method*

Accurately measured aliquots containing 1-10 mg of RCT from its standard solution (2 mg/mL) in methanol were introduced into 10-mL volumetric flasks and diluted to volume with methanol. Twenty µL of each solution were applied to a thin-layer chromatography (TLC) plate precoated with 0.25 mm silica gel F254 (20 × 10 cm; Fluka, Switzerland) using a microsyringe and developed in a mobile phase of isopropanol-ammonia (33%)-*n*-hexane (9:0.5:20, v/v/v). The plate was removed and air dried, and spots were scanned at 232 nm using the Densitometer-Dual Wave Flying Spot CS-9301 (Shimadzu, Kyoto, Japan). The calibration curve representing the recorded area under the peak and the corresponding concentration were plotted and the regression equation was computed.

2.4.3. *D_1 spectrophotometric method*

Aliquots of standard solution (0.1 mg/mL) equivalent to 0.05-0.4 mg of RCT were transferred to a series of 10-mL volumetric flasks filled to the mark with methanol. Using the UV-Vis Spectrophotometer 1601 (Shimadzu), D_1 spectra were recorded using methanol as a blank with $\Delta\lambda = 2$ and scaling factor of one. The calibration curve of trough height at 240 nm was plotted with respect to the drug concentration and the regression equation was calculated.

2.5. *Assay of prepared intact and degraded mixtures*

2.5.1. *HPLC method*

Different volumes of standard drug solution (0.1 mg/mL) in methanol equivalent to 0.36-0.04 mg RCT were transferred into a series of 10-mL volumetric flasks containing volumes of degraded solutions equivalent to degradation products derived from 0.04-0.36 mg. Volume was completed to mark with methanol, then 20 µL of each solution was chromatographed by HPLC method as described above.

2.5.2. *Densitometric method*

Volumes equivalent to 1-9 mg of RCT from its standard methanolic solution (2 mg/mL) were transferred to a series of 10-mL volumetric flasks, and then volumes of RCT degradation products derived from 1-9 mg of the drug were added. Each flask was filled to the mark with methanol and then analyzed by densitometric method as described above.

2.5.3. *D_1 spectrophotometric method*

Aliquots equivalent to 0.35-0.05 mg of RCT from its methanolic solution (0.1 mg/mL) were transferred to a series of 10-mL volumetric flasks. Different portions from an alkaline hydrolyzed solution equivalent to the degradation products were derived from 0.05-0.35 mg of the drug. The volume was completed with methanol and assayed by D_1 spectrophotometry at 240 nm using the D_1 spectrophotometric method described above.

2.6. *Analysis of acetorphan packets*

The contents of 5 acetorphan packets were thoroughly mixed. An amount of powder equivalent to 100 mg RCT

was weighed and dissolved in 40 mL of methanol by shaking in an ultrasonic bath for 10 min. The solution was filtered into a 50-mL volumetric flask and volume was completed with methanol to obtain a solution labeled to contain 2 mg/mL RCT for use with the densitometric method. The quantitative portion was then diluted with methanol to provide a solution labeled to contain 0.1 mg/mL RCT for analysis by the HPLC and D_1 spectrophotometric methods. Each method was assayed as described above and the concentration of the drug was calculated from the corresponding regression equation.

3. Results and Discussion

RCT, an enkephalinase-inhbitor, contains both ester and amide groups that are subject to hydrolysis by both acids and alkalies. Stressed hydrolytic degradation was performed to study RCT stability in acidic and alkaline media *via* refluxing in different concentrations of NaOH and HCl at different time intervals. Testing with TLC revealed that the drug was completely degraded after about 2 h using 0.1 N NaOH or HCl. Solutions were then neutralized using 0.5 N NaOH or 0.5 N HCl, evaporated to dryness under a vacuum, and extracted with methanol. Methanolic solutions were separated by TLC to produce three degradation products with almost the same retention times under both acidic and alkaline conditions. Alkaline degradation was thus used with the three methods to subsequently indicate the stability of the drug. A proposed pathway of alkaline hydrolysis under this condition is shown in Scheme 1.

3.1. *HPLC method*

Chromatographic separation of RCT and its degradation products was performed satisfactorily using an Eclipse C_{18} column. Separation was done several times to ascertain the optimum composition of the mobile phase using different solvents with different ratios, *i.e.*, acetonitrile-methanol (35:50, v/v) and acetonitrile-methanol-H_2O (50:25:15, v/v/v). The best separation was achieved with acetonitrile-methanol-water-acetic acid (52:28:20:0.1, v/v/v/v). Different flow rates (0.5-1.5 mL/min) were tested. Resolution of the intact and degraded drug was obtained at a flow rate of 0.7 mL/min. More than one wavelength was used, and the most sensitive detector response was obtained at 232 nm. Under these optimum conditions, pure RCT exhibited a sharp peak at 3.49 min, while its degradation products readily exhibited three peaks at 2.45, 2.86, and 5.48 min (Figures 1a and 1b). None of these peaks appeared in the chromatogram of the standard, indicating that the three identified peaks are due to degradation. Figure 1c represents a mixture of intact and degraded RCT, clearly indicating successful resolution of the intact peak and allowing the HPLC

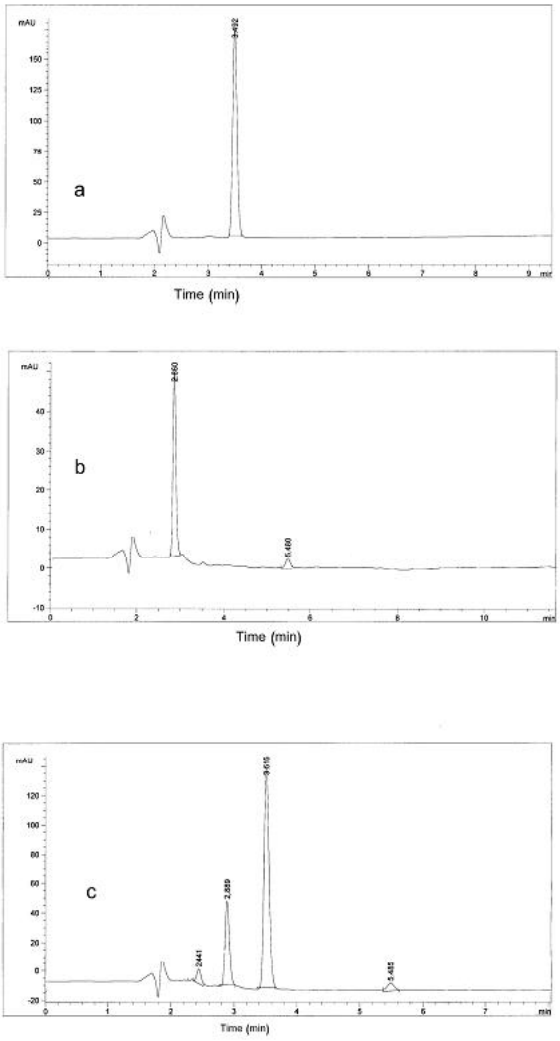
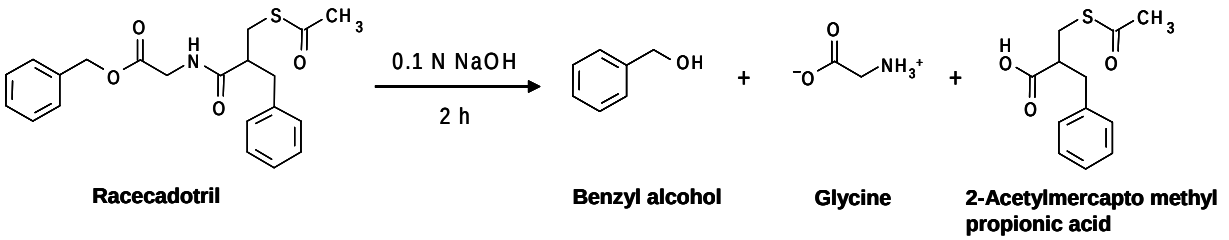


Figure 1. HPLC chromatogram at 232 nm. (**a**) RCT (40 µg/mL). (**b**) Degraded RCT (derived from 40 µg/mL). (**c**) Mixture of intact RCT and its degradation products (12:28 µg/mL).



Scheme 1. Proposed alkaline hydrolytic pathway of RCT.

method to be used to indicate the stability of the drug.

3.2. *Densitometric method*

The TLC densitometric method was used to determine RCT in the presence of its degradation products oknin accordance with differences in their R_f values. Different developing systems such as isopropanol-chloroform-ammonia (33%) (40:10:2, v/v/v), *n*-hexane-ammonia-methanol (10:1:30, v/v/v), and *n*-hexane-isopropanol-methanol-ammonia (20:20:30:1, v/v/v/v) were attempted, but complete separation of the drug from its degradation products was achieved using a mobile phase of isopropanol-ammonia (33%)-*n*-hexane (9:0.5:20, v/v/v) (Figure 2). The R_f value of the pure drug was 0.71, but the R_f value of its three degradation products was 0.09, 0.65, and 0.85, respectively.

3.3. *D_1 spectrophotometric method*

Zero-order absorption spectra of RCT and its degradation products resulted in overlapping that would interfere with direct determination of the drug, as shown in Figure 3. Derivative spectroscopy proved to be a simple and powerful technique for dealing with such an overlap. Examination of the first derivative D_1 spectrum of RCT and its degradation products revealed that the intact drug can be determined selectively using the trough at 240 nm. A zero-crossing point was indicated for the degradation products (Figure 4).

3.4. *Method validation*

3.4.1. *Linearity*

Using the suggested methods, a linear correlation was obtained between peak areas and the corresponding drug concentration in the range of 4-40 µg/mL for the HPLC method. With the densitometric method, a linear relationship between peak areas of the separated spots and the corresponding RCT concentration was in the range of 2-20 µg/spot. Moreover, linearity between the trough amplitude of the D_1 curve at 240 nm and the corresponding drug concentration was obtained in the range of 5-40 µg/mL for the derivative method. The characteristic parameters of regression equations and correlation coefficients were calculated and are listed in Table 1.

3.4.2. *Accuracy and precision*

The three proposed methods were tested three times; accuracy ranged between 99.2-99.5 ± 0.56-1.02% for

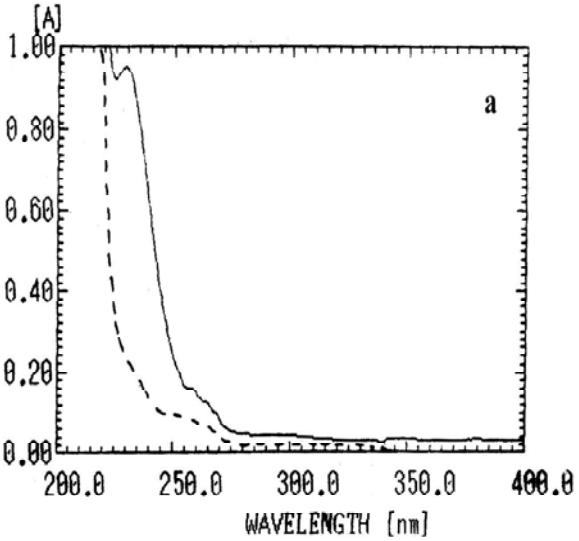


Figure 3. Absorption spectra of 100 µg/mL intact racecadotril (—) and 100 µg/mL of its degradation products (---) in methanol.

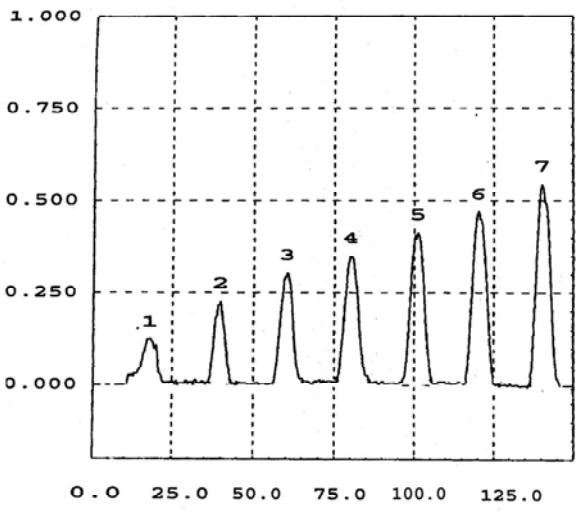


Figure 2. Densitometric chromatogram of RCT (2-20 µg per spot) at 232 nm.

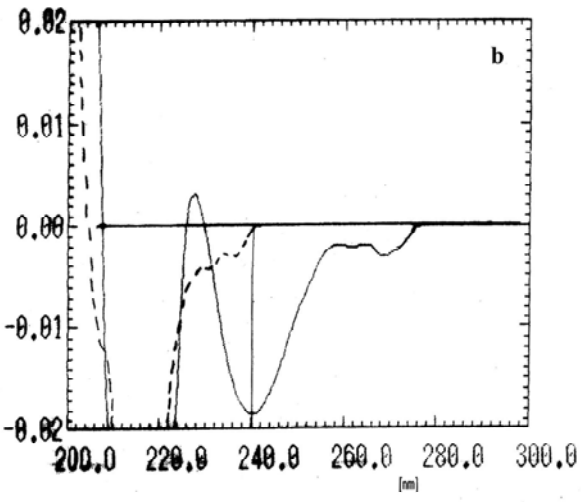


Figure 4. First derivative spectra of 40 µg/mL intact racecadotril (—) and 40 µg/mL of its degradation products (---) in methanol.

RCT with three concentrations within the linearity range. Precision was also evaluated by calculating the intraday RSD%, which ranged between 0.33 and 0.84% and was found to be 0.34-0.98% over a period of two months. This indicated the repeatability and reproducibility of the proposed methods (Table 1).

3.4.3. *Specificity*

Laboratory prepared mixtures containing different percentages of the drug and its degradation products were analyzed. The three methods were valid at determining the pure drug in the presence of up to 90% of its degradation products without any interference; as shown in Table 2, recovery was satisfactory in a range of 99.9-100.8 ± 0.55-0.82%, and the methods were successful at indicating stability.

The specificity of the proposed methods was further evaluated by successful analysis of the drug in its pharmaceutical formulation. With acetorphan packets, the HPLC, densitometric, and D_1 methods had mean recovery of 101.3 ± 1.68, 99.5 ± 0.56, and 101.5 ± 1.59%, respectively (Table 3). The results obtained were reproducible with a low relative standard deviation of no more than 1.7%. These results were compared with those obtained with the reported direct UV spectrophotometric method (*4*). As shown in Table 3, calculated *t*- and *F*-values were less than theoretical

Table 1. Regression parameters and assay validation results for the determination of RCT by the proposed methods

Parameters	HPLC method	Densitometric method	D_1 spectrophotometric method
Linearity range	4-40 μg/mL	2-20 μg/spot	5-40 μg/mL
Regression parameters			
Slope ± S.D.	23.889 ± 7.24	169.970 ± 2.04	0.0370 ± 0.001
Intercept ± S.D.	19.293 ± 8.86	254.651 ± 23.77	-0.041 ± 0.02
S.D. of residual	11.379	33.944	0.032
Correlation coefficient	0.9997	0.9995	0.9996
Accuracy (R% ± S.D.)	99.5 ± 0.88	99.5 ± 0.56	99.2 ± 1.02
Precision (RSD%, *n* = 9)			
Intraday	0.51-0.73	0.80-0.84	0.33-0.42
Interday	0.69-0.98	0.34-0.70	0.60-0.89

Table 2. Determination of RCT in mixtures with its degradation products using the proposed methods

HPLC method			Densitometric method			D_1 spectrophotometric method		
Intact (μg/mL)	Degraded (μg/mL)	R% of intact	Intact (μg/mL)	Degraded (μg/mL)	R% of intact	Intact (μg/mL)	Degraded (μg/mL)	R% of intact
36	4	99.9	18	2	99.9	35	5	100.7
28	12	101.3	14	6	100.9	28	12	99.8
20	20	101.5	10	10	100.4	20	20	99.3
12	28	99.7	6	14	100.2	12	28	100.4
8	32	101.6	4	16	101.1	8	32	98.9
4	36	100.8	2	18	99.7	5	35	100.5
Mean ± S.D.		100.8 ± 0.82			100.4 ± 0.55			99.9 ± 0.72

Table 3. Determination of RCT in acetorphan packets by the proposed methods in comparison to the reported method (*4*)

Parameters	HPLC method	Densitometric method	D_1 spectrophotometric method	Reported method (*Ref. 4*)
Mean%	101.3	99.5	101.5	99.6
S.D.	1.68	0.56	1.59	0.92
Variance	2.84	0.32	2.54	0.83
N	5	5	5	5
t-test	1.89	0.25	1.43	
F-test	3.42	2.59	3.06	
Standard addition				
Mean ± S.D.%	98.6 ± 1.07	100.1 ± 1.02	100.5 ± 0.88	

The theoretical *t*- and *F*-values at $p = 0.05$ were 2.31 and 6.39, respectively. The reported method (*4*) is UV measurement of the drug at 231 nm in methanol.

ones, indicating that there was no significant difference between the proposed and reported methods with respect to accuracy and precision.

Validity of the proposed methods was further assessed using the standard addition technique; mean recovery of the added amount was 98.6 ± 1.07%, 100.1 ± 1.02%, and 100.5 ± 0.88% for the three methods, respectively (Table 3).

4. Conclusion

The suggested methods were the first to indicate stability for the determination of RCT in its bulk powder or pharmaceutical formulation without interference from its degradation products or excipients. In addition, they are simple, rapid, accurate and precise and can be used for routine analysis in quality control laboratories.

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