# SPECTROPHOTOMETRIC AND SPECTROFLUORIMETRIC METHODS FOR DETERMINATION OF RACECADOTRIL

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## ABSTRACT

Two accurate and sensitive spectrophotometric and spectrofluorimetric methods were developed for determination of Racecadotril. In the first method reduction of $Fe^{3+}$ into $Fe^{2+}$ in presence of o-phenanthroline by Racecadotril to form a stable orange-red ferroin chelate $[Fe\text{-}(Phen)_3]^{2+}$ was the basis for its determination . The absorbance at 510 nm was measured and linear correlation was obtained in the concentration range of 2.5 – 25 µg $mL^{-1}$. In the second method the native fluorescence of Racecadotril in acetonitrile solvent at $\lambda = 319$ nm when excitation was at 252 nm is used for its determination. Linear correlation was obtained in the concentration range of 50 to 500 ng $mL^{-1}$. The proposed methods were applied for determination of Racecadotril in bulk powder with mean accuracy of 100.39±1.239 for the spectrophotometric method and 100.09± 1.042 for the spectrofluorimetric method. The proposed methods were successfully applied for determination of Racecadotril in its pharmaceutical dosage form.

**Keywords**: Spectrophotometric; spectrofluorimetric; racecadotril.

## INTRODUCTION

Racecadotril (RAC), (±)-benzyl 2-(2-(acetylthiomethyl)-2-methyl-3 phenylpropanamido) acetate (fig. 1) is a new anti-diarrheal drug (The Merck Index, 2006).

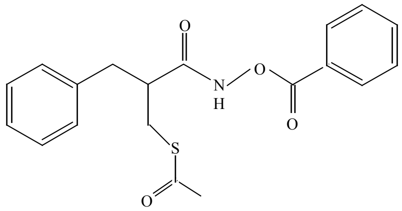

**Fig. 1**: Chemical structure of Racecadotril.

In peripheral tissue membranes, RAC is converted into thiorphan, which inhibits the enzyme enkephalinase. Encephalin concentration is increased as a result of this, leading to activation of opioid receptors and a decrease in cAMP level. This in turn results in reduced secretion of water and electrolytes into the intestinal lumen (Vetel *et al.,* 1999 and Lecomte, 2000).

RAC and its metabolite, thiorphan in human plasma have been determined by LC with UV detection after Solid-phase extraction (Fan *et al.,* 2008). RAC has been also determined by RP-LC with gemfibrozil as internal standard (Prabhu *et al.,* 2007). Three unknown impurities in RAC bulk drug at levels below 0.5% have been detected by simple reversed-phase isocratic LC method. The structures of these impurities have been proposed based on molecular ion information (Reddy *et al.,* 2006). Quantitative analysis of RAC, in raw material and in capsules has been achieved by UV spectroscopy and RP-LC (Rao and Nappinnai, 2007; Vetrichelvan and Prabakaran, 2007). A liquid chromatographic–tandem mass spectrometric method for quantification of thiorphan in human plasma, using lisinopril as internal standard, has been developed and validated (Yu *et al.,* 2007). Stability indicating methods for the determination of RAC in presence of its degradation products were developed including HPLC, TLC and derivative spectroscopy (Mohammed et al., 2009).

Spectrophotometric methods are considered the methods of choice used on a large scale in pharmaceutical industry especially in quality control laboratories. Nevertheless, no colorimetric or fluorimetric method has been reported so far for determination of RAC.

Therefore, the objective of this work is to develop accurate, sensitive and reliable spectrophotometric and spectrofluorimetric methods for determination of RAC either in bulk powder or in pharmaceutical formulation.

## EXPERIMENTAL

### *Apparatus*

1. Double beam UV-Visible spectrophotometer (SHIMADZU, Japan) model UV-1601 PC with quartz cell of 1 cm path length. The software used was UVPC personal spectroscopy software version 3.7. Spectral bandwidth was 2 nm and wavelength-scanning speed was 2800nm/min.
2. Digital pH meter (JENCO, Electronics, Ltd, U.S.A, Model 671P).

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3. Thermostatic water bath (PolyScience®, TUV, Model 20L-M).
4. Jasco FP-6200 Spectrofluorimeter equipped with xenon lamp and 1 cm quartz cuvette (Japan). [*Band width (Excitation): 5 nm, Band width (Emmission): 20 nm,-Response: 0.02 sec, Sensitivity: medium, Scanning speed: 500 nm.min$^{-1}$*].
5. Sonix TV ss-series ultrasonicator (USA).

### *Materials*

*Pure samples*

RAC was kindly provided by EPSI pharmaceuticals, Beni-Sueif, Egypt. The purity of the sample was checked and found to be 100.10±1.238% according to the company method (an HPLC method in which isocratic elution of 100 μg.mL$^{-1}$ RAC was done using ammonium acetate (pH=5): acetonitrile (6:4) as the mobile phase at a flow rate of 1.2 mL min$^{-1}$ and UV detection at 210 nm).

*Market samples*

Acetorphan® sachets (Batch No. 708912) (EPSI, Beni-Sueif, Egypt): each sachet is claimed to contain 300 mg of RAC.

*Reagents*

All reagents and chemicals used were of analytical grade and were used without further purification

1. 1,10-phenanthroline monohydrate (Merck, Darmstadt, Germany).
2. Ammonium ferric sulphate dihydrate [$(NH_4)$ Fe $(SO_4)_2.2H_2O$], (El-Nasr Pharmaceutical Chemicals Co., Abu-Zabaal, Cairo, Egypt).
3. Ferric-phenanthroline reagent, prepared by dissolving 0.2 gm of 1,10-phenanthroline monohydrate and 0.16 gm of ammonium ferric sulphate in 2 ml 1M hydrochloric acid and dilution to 100 ml with water.
4. Acetate buffer pH range of 3.4 - 6, prepared by mixing appropriate volumes of 0.2M acetic acid and 0.2M sodium acetate (The British Pharmacopeia, 2007).
5. Methanol HPLC grade (Sigma Aldrich, Germany).
6. Hydrochloric acid (E.Merck, Germany).
7. Acetonitrile HPLC grade (E.Merck, Germany).
8. Deionized water (SEDICO pharmaceutical Co., 6th October City, Egypt).

*Standard solutions*

1. Stock standard solution (1 mg mL$^{-1}$): weigh accurately 0.1 gram of RAC into a 100-ml volumetric flask, add 50 ml methanol and shake to dissolve, then complete the volume with methanol.
2. Working standard solutions:
   (1) *For the spectrophotometric method* (100 μg ml$^{1-}$): transfer accurately 10 ml of RAC stock standard solution (1 mg.mL$^{-1}$) into a 100-ml volumetric flask, then complete the volume with methanol.
   (2) *For the spectrofluorimetric method* (1 μg ml$^{-1}$): transfer accurately 1 ml of RAC working standard solution (1) into a 100-ml volumetric flask, then complete the volume with methanol.

## Methods

*Linearity*

1. *For the spectrophotometric method*: Transfer accurate measured aliquots equivalent to 25-250 μg of RAC from its working standard solution (1) into a set of 20-mL Stoppard tubes containing 5 mL acetate buffer (pH=4.4±0.2), add 1.5 mL ferric-phenanthroline reagent to each tube. Place into a thermostatic water bath at 100ºC for 45 min. Cool immediately to room temperature. Transfer the content of each tube quantitatively into a set of 10-mL volumetric flasks. Complete the volume with water. Measure the absorbance at 510 nm. Construct the calibration curve relating the absorbance of the obtained colored solution at 510 nm to the corresponding RAC concentration and calculate the regression equation.
2. *For spectrofluorimetric method*: Transfer aliquots equivalent to 0.5-5 μg of **RAC** from its working standard solution (2) into a set of 10-mL volumetric flasks .Complete the volume with acetonitrile. The fluorescence intensity was recorded at $\lambda_{em}$ = 319 nm using $\lambda_{ex}$= 252 nm. Construct the calibration curve relating the fluorescence intensity /100 at $\lambda_{em}$ = 319 nm to the corresponding concentrations and calculate the regression equation.

A*pplication of the proposed methods to pharmaceutical dosage form*:

1. *For the spectrophotometric method*: The contents of ten sachets of Acetorphan® were thoroughly mixed then an amount of the powder equivalent to 100 mg of RAC was weighed accurately into a 250-mL beaker, 70 mL of methanol was added, stirred magnetically for about 30 min then filtered through filter paper into a 100- mL volumetric flask, the beaker and the funnel were washed and the volume was completed with methanol. Appropriate dilution was made to bring up concentrations of 5 and 10 μg mL$^{-1}$ of RAC. The proposed spectrophotometric method was applied for the analysis and calculation of RAC concentration.
2. *For the spectrofluorimetric method:* The above procedure was repeated but appropriate dilution was made to bring up a concentration of 1μg.mL$^{-1}$ of RAC. Further dilution was made to bring up concentrations of 100 and 200 ng mL$^{-1}$ of RAC using acetonitrile as solvent. The proposed spectrofluorimetric method was applied for the analysis and calculation of RAC concentration.

## RESULTS

### *Method development*

*Spectrophotometric method*

In this method, RAC reduces ferric-phenanthroline (ferriin) to ferrous-phenanthroline (ferroin) where the obtained ferroin chelate has an orange-red color that can be measured colorimetrically at 510 nm (fig. 2).

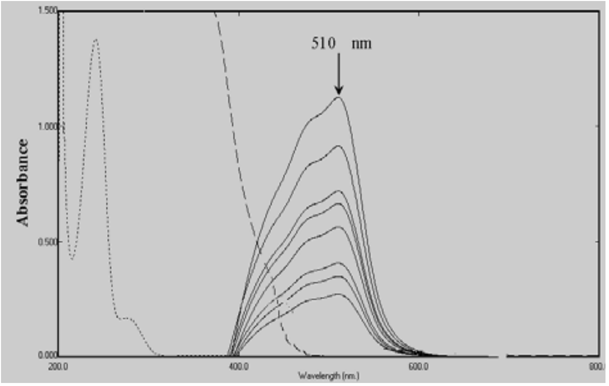

**Fig. 2**: Absorption spectra of Racecadotril/ferric-phenanthroline reaction product in the range of 2.5–25 µg $mL^{-1}$ (—— ), 25 µg $mL^{-1}$ of Racecadotril (………) and reagent blank (-------).

A linear relationship was obtained between the absorbance value at 510 nm and RAC concentration in the range of 2.5-25 µg $mL^{-1}$. The regression equation was calculated and found to be:

$Y= 0.037\ C + 0.157$, $r = 0.9998$

Where Y is the absorbance at 510 nm, C is RAC concentration in µg $mL^{-1}$ and r is the correlation coefficient.

*Spectrofluorimetric method*

RAC showed native fluorescence in acetonitrile solvent at $\lambda$ = 319 nm when excited at 252 nm (fig. 3). A linear calibration curve was obtained in the range of 50-500 ng $mL^{-1}$.

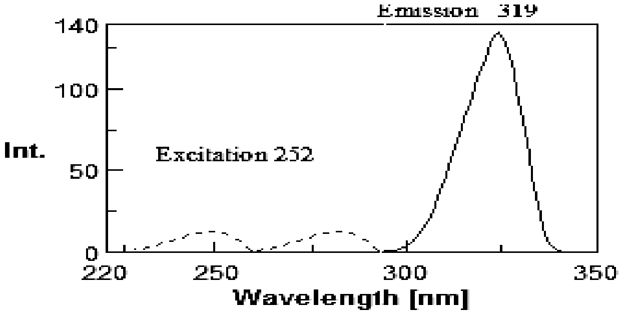

**Fig. 3**: Emission spectrum ($\lambda_{ex}$=252 nm) and excitation spectrum ($\lambda_{em}$=319 nm) of RAC [50 ng $mL^{-1}$] in acetonitrile.

A linear relationship was obtained between the intensity of fluorescence /100 of RAC at 319 nm, and concentrations of RAC in the specified above range. The regression equation was calculated and found to be:

$F= 0.0027\ C + 0.1607$, $r = 0.9992$

Where F is the fluorescence intensity/100 of RAC at 319 nm, C is the concentration of RAC in ng $mL^{-1}$ and r is the correlation coefficient.

### *Method optimization*

*Spectrophotometric method*

The effect of reagent volume was studied over a range of 0.5-4 ml where maximum color intensity was obtained upon using 1.5ml of ferric-phenanthroline reagent.

The effect of buffer pH on ferroin chelate absorbance was checked over a range of 3.6-6, where the absorbance at 510 nm remained stable between pH 3.6-4.8. Above and below this range the absorbance decreases may be due to the incomplete reduction of ferric-phenanthroline reagent.

The volume of acetate buffer pH = 4.4±0.2 was examined and the maximum absorbance at 5 ml acetate buffer was found to be optimum for maximum color intensity and minimum blank absorbance.

Different temperatures were tested using thermostatic water bath set at 25, 50, 75, 80, 90 and 100ºC, where maximum color development was obtained at 100ºC.

The solutions were maintained for variable time intervals in a boiling water bath at 100ºC. The maximum stable absorbance was obtained after 45 minutes. The effect of time of standing on the absorbance revealed that the color developed was stable for at least 2 hours without change in its intensity

*Spectrofluorimetric method*

Different solvents were tested e.g. dil. $H_2SO_4$, dil. HCl, 0.1 N NaOH, methanol, ethanol, propanol, $CHCl_3$, acetonitrile and water where acetonitrile was found to be the most suitable solvent that provides maximum fluorescence intensity.

Different excitation wavelengths were tested where maximum fluorescence intensity was obtained by excitation at 252 nm.

Different surfactants were tried, namely, tween 60, tween 80, span, but no effect on fluorescence intensity was evident.

The effect of time on the stability of fluorescence intensity was studied and RAC fluorescence intensity at 319 nm was found to be stable over 1 hour in acetonitrile solvent.

### *Method validation*

The linearity of the calibration graphs is apparent from the high value of the correlation coefficient and the low intercept (table 1). Results from determination of precision, LOD and LOQ are also given in table 1 and the good recovery and thus accuracy as well as specificity of the proposed methods are apparent from the results listed in table 2. The robustness of the method was evident as the effect of different factors on color development in case of spectrophotometric method and on fluorescence

**Table 1**: Results of assay validation parameters of the proposed methods for the determination of RAC

| Parameters | Spectrophotometric method | Spectrofluorimetric method |
|---|---|---|
| Range µg mL$^{-1}$ | 2.5-25 µg mL$^{-1}$ | 50-500 ng mL$^{-1}$ |
| Slope | 0.037 | 0.0027 |
| Intercept | 0.157 | 0.1607 |
| Correlation coefficient (r) | 0.9998 | 0.9992 |
| Accuracy (mean ± SD) | 100.39±1.239 | 100.09± 1.042 |
| Precision (RSD%) | 1.472 | 0.343 |
| Repeatability[a] | 0.412 | 0.876 |
| Intermediate precision[a] | 0.853 | 14.67 ng mL$^{-1}$ |
| LOD | 0.80µg mL$^{-1}$ | 44.44 ng mL$^{-1}$ |
| LOQ | 2.43 µg mL$^{-1}$ | 50 – 500 ng mL$^{-1}$ |

[a]The intra-day and inter-day relative standard deviations of the average of concentrations 5, 10 and 15 µg mL$^{-1}$ (for spectrophotometric method) and 50, 100, 150 ng mL$^{-1}$ (for spectrofluorimetric method) of RAC.

**Table 2**: Application of standard addition technique to the analysis of RAC by the proposed methods

| Taken (µg mL$^{-1}$) Acetorphan sachet Batch No. 708912 | Spectrophotometric method | | Spectrofluorimetric method | | Pure added (µg mL$^{-1}$) | Spectrophotometric method | | Spectrofluorimetric method | |
|---|---|---|---|---|---|---|---|---|---|
| | Found[a] (µg mL$^{-1}$) | Re-covery % | Found[a] (µg mL$^{-1}$) | Re-covery % | | Pure found[b] (µg mL$^{-1}$) | Re-covery % | Pure found[b] (µg mL$^{-1}$) | Re-covery % |
| 5.00 | 4.96 | 99.20 | | | 5.00<br>10.00<br>15.00 | 5.12<br>10.08<br>15.14 | 102.40<br>100.80<br>100.93 | | |
| Mean± S.D. | | | | | | | 101.38<br>±0.889 | | |
| 100.00 | | | 100.03 | 100.03 | 50.00<br>100.00<br>150.00 | | | 49.00<br>97.00<br>148.50 | 98.00<br>97.00<br>99.00 |
| Mean± S.D. | | | | | | | | | 98.00<br>±1.000 |

[a] average of six experiments, [b]average of three experiments

intensity in case of spectrofluorimetric method was studied to obtain the optimum parameters for conducting both methods.

## DISCUSSION

*Spectrophotometric method*

In this method, a simple and accurate spectrophotometric method using Fe(III)-phenanthroline reagent was developed for determination of RAC. Fe(III) ion plays a prominent role in the spectroscopic determination of many pharmaceutical compounds acting as an oxidant; ferric ion is reduced to ferrous form, the latter can be determined using 1,10-phenanthroline (Nagaralli *et al.,* 2002). Ferric-phenanthroline is a highly chromogenic reagent, used for the determination of phenolic compounds (Koch *et al.,* 1992), ascorbic acid (Sultan *et al.,* 1994), and some antibacterial and non-steroidal anti-inflammatory drugs (Nagaralli *et al.,* 2002), itraconazole (Murthy *et al.,* 2002), amlodipine (Rahman *et al.,* 2004), cephalosporins (Al-Momani, 2001). RAC was suggested to have a reducing character, which allows its determination by ferric-phenanthroline**.**The reaction conditions were optimized in order to maximize color intensity and drug sensitivity. The effect of different factors on color development including, volume of reagent, pH of buffer, volume of buffer, effect of temperature, time of heating and stability of color was studied.

*Spectrofluorimetric method*

In this method, RAC showed native fluorescence in acetonitrile solvent at λ = 319 nm when excited at 252 nm which can be used for its determination.

The robustness of the methods was evident as the effect of different factors on color development in case of spectrophotometric method and on fluorescence intensity in case of spectrofluorimetric method was studied to btain the optimum parameters for conducting both methods.

**Table 3**: Statistical comparison of results obtained by the proposed methods and the reference method

| Data | Spectrophotometric method | Reference method [a] | Spectrofluorimetric method |
|---|---|---|---|
| Mean % | 99.20 | 100.10 | 100.03 |
| SD | 1.113 | 1.238 | 1.023 |
| N | 6 | 6 | 6 |
| Student 's t-test (2.23)[b] | 0.406 | | 0.015 |
| *F(5.05)*[b] | 1.001 | | 1.412 |

[a] EPSI HPLC method. [b] Figures in parenthesis represent the corresponding tabulated values for *t* and *F* at *P*=0.05.

## CONCLUSION

O-Phenanthroline is a sensitive chromogenic reagent for the determination of RAC and up to 2.5-25 µg mL$^{-1}$ of RAC can be determined either in pure form or in pharmaceutical formulation using this reagent. The spectrofluorimetric method is more sensitive since up to 50 ng. mL$^{-1}$ of RAC can be determined using this technique.

Statistical analysis was performed by comparing the results of the proposed procedures with those of manufacturer method. No significant difference was observed regarding accuracy and precision, as shown in table 3.

The suggested methods provide accurate, selective and sensitive analytical procedures for the determination of RAC. They are suitable for routine analysis and quality control of RAC in its pharmaceutical formulation.

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