Forced Degradation Study of Racecadotril: Effect of Co-solvent, Characterization of Degradation Products by UHPLC-Q-TOF-MS/MS, NMR and Cytotoxicity Assay

Vishnuvardhan Chiguru [1], Allakonda Lingesh [3], Srinivas R. [1,*], Satheeshkumar N. [1]

[1] Department of Pharmaceutical Analysis, National Institute of Pharmaceutical Education and Research, Hyderabad (NIPER-H), Balanagar, Hyderabad 500037 Telangana, India

[2] National Center for Mass Spectrometry, CSIR-Indian Institute of Chemical Technology, Tarnaka, Hyderabad 500607 Telangana, India

[3] Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research, Hyderabad (NIPER-H), Balanagar, Hyderabad 500037 Telangana, India
* Corresponding Author. E-mail: srini@iict.res.in

Abstract. Racecadotril, an enkephalinase inhibitor, was subjected to hydrolysis (acidic and alkaline), oxidation, photolysis and thermal stress, as per ICH specified conditions. The drug showed extensive degradation under acidic, basic hydrolysis and oxidative stress conditions whereas, it was stable under other stress conditions. A total of seven degradation products (DPs) were observed. The chromatographic separation was optimized on Acquity HSS Cyano (100 × 2.1 mm, 1.8 µ) column using 0.1% formic acid and acetonitrile as mobile phase in gradient mode. Six DPs were characterised by LC–MS/MS and DP1 by GC–MS. The major DPs (DP 2 and DP 5) were isolated and characterised by NMR. This is a typical case of degradation where co solvent methanol reacts with racecadotril leading to the formation of pseudo DPs, DP 6 and DP 5. Interestingly the MS/MS spectra of protonated drug, DP 4 and DP 7 showed product ions which were formed due to intramolecular benzyl migrations. In vitro cytotoxic activity studies on isolated DP 2 and DP 5 revealed that the former has no cytotoxic nature, whereas the latter has potential pulmonary and hepatic toxicity.

Keywords: Forced degradation; LC–MS/MS; Benzyl-benzyl interactions; GC–MS; Cytotoxic assay.

1. Introduction

Racecadotril (RACE) [(RS)-benzyl N-[3-(acetylthio)-2-benzylpropanoyl] glycinate] is an antidiarrheal drug which acts by inhibiting enkephalinase peripherally and suitable for use in infants and young children. It is rapidly converted in the body to thiorphan, a potent enkephalinase inhibitor. Enkephalins are endogenous opioid peptides secreted by myenteric and sub mucosal neurons in the digestive tract. The mechanism of action of enkephalins involves activation of the δ opioid receptor which inhibits the secretion of chlorine ions and fluids leading to the loss of fluids and electrolytes during diarrhea [1], [2] and [3]. Stress stability is an integral part of the drug development process and explains several factors that affect the expiration dating of drug products, including the chemical and physical stability during the preclinical formulation stages, process development, packaging development, and post marketing life. Benefits of structural characterization of degradation products includes, understanding of their origin resulting into ways for their control during drug synthesis or formulation development. In addition, the degradation products can either be isolated or be synthesized and subsequently their toxicity can be evaluated with the help of cytotoxicity tests like MTT assay which helps in drug discovery and development research [4].

A few analytical methods have been reported on RACE, which includes stability indicating assay methods [5], [6], [7] and [8], bioanalytical methods [9] and [10]. The hydrolytic degradation behavior of RACE was studied under acidic and alkaline conditions by Pawan et al. [11]. However, no systematic study on the degradation behavior of racecadotril and characterization of all the DPs

formed has been reported. In general stress studies are limited to non-volatile DPs, whereas the identification and characterization of volatile DPs is not done in routine. The study of volatile DPs along with non-volatile DPs also helps in establishment of mass balance. Hence, the purpose of the present study is to develop a stability indicating assay method for RACE and to characterize the degradation products (volatile and non-volatile) formed as per the International Conference on Harmonization (ICH) recommended stress conditions using LC-ESI–MS/MS, GC–MS, NMR and cytotoxicity evaluation of isolated major degradation products using MTT assay. The results of the present study may also be helpful for the assessment of the quality of stored products that have expired or on the edge of getting expiration.

2. Experimental

2.1. Drug and reagents

Pure racecadotril (99.56% purity) was obtained as a gift sample from Symed Labs Hyderabad, India. UPLC grade methanol, acetonitrile and hydrogen peroxide were purchased from Merck (Mumbai, India). All analytical grade reagents: ammonium formate, formic acid, sodium hydroxide, hydrochloric acid, and 30% (w/w) hydrogen peroxide were purchased from SD Fine Chemicals Pvt. Ltd., (Mumbai, India). LC grade water was prepared by filtrating through a Millipore Milli-Q- plus system (Merck Millipore, Billerica, Massachusetts, United States). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from Sigma–Aldrich. Neuro-2a (mouse neuroblastoma cells), A549 (adenocarcinomic human alveolar basal epithelial cells) and Hep G2 (human liver carcinoma cell) cells were obtained from American Type Culture Collection (Manassas, VA, United States).

2.2. Instrumentation

For LC–MS analysis, an Agilent Infinity 1290 series instrument (Agilent Technologies, Santa Clara, California, USA) coupled to quadrupole time-of-flight (Q-TOF LC–MS 6540) equipped with an electrospray ionisation (ESI) source was used. The data acquisition was under the control of Mass Hunter workstation software. The ESI source conditions were optimized as follows: fragmentor voltage, 150 V; capillary voltage, 4000 V; skimmer, 65 V; nitrogen was used as drying (350 °C; 10 L/min) and nebulising (40 psi) gas. For full scan MS mode, the mass range was set at m/z 50–2000. Ultra-high purity nitrogen was used as collision gas. All the spectra were recorded under identical experimental conditions and at an average of 20–25 scans.

Identification of volatile degradation product was carried out on an Agilent 6890 gas chromatograph (Agilent Technologies Inc., Santa Clara, California, USA) equipped Mass Selective Detector (MSD, 5973), autosampler (G2614), and electron impact (EI) ionisation source controlled by ChemStation software.

1D and 2D NMR experiments were performed on a 500 MHz NMR (AVANCE III HD-500, Bruker, Billerica, Massachusetts, United States) spectrometer using CDCl3 as solvent. 1H chemical shift values were reported on the δ scale in ppm relative to TMS (δ = 0.00 ppm) as internal standard. The data acquisition and processing of NMR spectra was done using Top spin software (3.2 version).

Photolytic studies were carried out in a photostability chamber (Osworld OPSH-G-16-GMP series, Osworld Scientific Equipments Pvt. Ltd. India) set at 40.0 ± 5.0 °C/75.0% relative humidity (RH) ±3.0% RH and equipped with an illumination bank on inside top, consisting of a combination of two black light ultraviolet lamps and four white fluorescent lamps in accordance with option 2 of the ICH guideline Q1B. The thermal degradation studies were carried out in the Osworld laboratory oven (Osworld scientific Pvt. Ltd. India). All pH measurements were carried out on a pH meter (Metrohm Schweiz AG, 780 pH meter, Germany) with Epson printer Lx-300 t and weighing was done on a Sartorius balance (CD 225 D, 22308105 Germany).

2.3. Forced degradation studies

Forced degradation of RACE was carried out on the bulk drug as per ICH guidelines [12] and [13]. RACE was subjected to stress hydrolytic degradation study by refluxing 30 min in acidic (1.0N HCl), and 5 min in alkaline (0.01N NaOH) conditions at room temperature using acetonitrile and methanol

as co solvents. The optimized oxidative, photolytic, and thermal stress conditions are given in Table 1. The drug solutions were prepared at 1.0 mg/mL concentration for all stress samples.

2.4. Sample preparation

All the stressed samples (hydrolytic, oxidative, thermal, and photolytic stress) were neutralized and diluted with mobile phase and filtered through 0.22 μ membrane filter before LC–MS analysis.

2.5. Cytotoxicity assay

Neuro-2a, A549 and Hep G2 cells were seeded at a density of 5 × 103 cells per well in 96-well plate. After 24 h incubation in CO2 incubator, DP 2 and DP 5 were added in different concentrations over a range of 3.125–100 μM, incubated further for 48 h. 100 μ L of MTT at 0.5 mg/mL concentration was added to the above sample and incubated for 4 h. Then the media with MTT was removed and the resulted formazan crystals were dissolved by addition of 100 μL DMSO and absorbance was measured at 570 nm using multi detection plate reader (Spectramax M4, Molecular devices, USA).

2.6. Isolation of major degradation products by column chromatography

For isolation of major degradation products (DP 1, DP 2 and DP 5) initially base hydrolytic condition was used, but the degradation products formed were not stable and within less time they are converted into DP 1. Therefore acidic hydrolytic condition was tried by dissolving 300 mg of RACE in 5 mL of methanol, and added 15 mL of 1N HCl. Final volume was made up to 25 mL with methanol and refluxed for 4 h at 80 °C, where DP 1, DP 2 and DP 5 were formed in major quantity. The DP 1 was volatile liquid and it got evaporated along with solvents (methanol and 1N HCl) and was received in collecting flask leaving remaining degradation products in rotating flask, which were solidified and loaded on to the silica column. Fractions containing>99% of DP 5 (eluted with 40% ethyl acetate in hexane), DP 2 (eluted with 70% ethyl acetate in hexane) were pooled together; concentrated on rotavapor to remove ethyl acetate and hexane using a rotary evaporator. DP 2 and DP 5 were obtained as white powder with chromatographic purity of 99.10% and 99.60% respectively.

3. Results and discussion

3.1. Chromatographic separation

The preliminary experiments were done to develop a chromatographic method capable of resolving the drug and its stress degradation products. Initial trials were performed on Acquity HSS SB C18 (100 × 2.1 mm, 1.8 μ) using 0.1% formic acid as aqueous phase and acetonitrile, methanol as organic phase in gradient elution mode. Better separation and peak shape were observed with formic acid and acetonitrile, but resolution for the critical pair, DP 4 and RACE was less (1.3). In order to improve the resolution of critical pair, instead of changing gradient method and mobile phase components, different columns i.e. Acquity HSS T3 (100 × 2.1 mm, 1.8 μ), Acquity CSH C18 (100 × 2.1 mm, 1.7 μ) and Acquity HSS Cyano (100× 2.1 mm, 1.8 μ) were tried and better separation was found with HSS Cyano column (resolution is 2). A linear gradient program was set as follows: 0/30, 2/30, 5/70, 7/70, 8/30, and 10/30 at flow rate of 0.3 mL/min. Chromatogram was monitored at 215 nm, column temperature was 30 °C and injection volume was 2 μL. With the developed method RACE and its degradants were well separated with adequate resolution and symmetric peak shape.

For characterisation of volatile DP, Zebron ZB-624 (Phenomenex) column (30 m × 0.25 mm × 1.40 μ) was used with an initial GC oven temperature of 35 °C (2 min hold). The temperature was raised at the rate of 8 °C/min to 220 °C, where it was maintained for 5 min; injector temperature was set at 220 °C and the MSD interface was set at 230 °C. The standard split/splitless liner was used in split injection mode (1:50). Hydrogen was used as carrier gas at a constant flow rate of 1.2 mL/min.

3.2. Degradation behavior of RACE

The degradation behavior of RACE was studied using LC–MS under various forced degradation conditions. Sufficient degradation was observed in all conditions except in photolysis and thermal degradation where drug was found to be stable. When methanol was used as co solvent in hydrolytic degradation (acidic and basic) pseudo DPs, DP 5 and DP 6 were formed. This may be attributed to participation of methanol in the degradation chemistry by acting as a nucleophile to react with

electrophilic sites or intermediates in the degradation pathways (Scheme S4a,b, Supporting information). The overlay of chromatograms of all stress degradation samples are given in Fig. 1.

3.3. Characterization of RACE and its DPs by LC–MS/MS

RACE and all the DPs (DP 1–7) were well separated by LC and they exhibited abundant protonated molecular ions ([M+H]+) in positive ionization mode. MS/MS spectra of the [M+H]+ ions of RACE and DPs were recorded to obtain structural information. The fragmentation patterns were obtained based on MS/MS experiments and accurate mass measurements from HRMS data. A total of seven DPs were formed but only six DPs were characterized using LC-ESI–MS/MS experiments as DP 1 was not detected under ESI, APCI and APPI ionisation conditions and the proposed structures of DPs and their elemental compositions are given in Scheme 1 and Table S1, Supporting information. However, DP-1 was analyzed by GC-EIMS which will be discussed later.

The ESI–MS/MS spectra of protonated RACE and those of DPs are shown in Fig. 2, and the proposed fragmentation patterns of their [M+H]+ ions are shown in Schemes S1–S4, Supporting information. The elemental composition of all the ions of RACE and the DPs are summarized in Table S1, Supporting information.

3.3.1. MS/MS of [M+H]+ of RACE (m/z 386)

To study the degradation behavior of RACE, the ESI–MS/MS spectrum of its protonated molecule (m/z 386) was examined. The spectrum showed product ions at m/z 344 (loss of ketene, CH2CO from m/z 386), m/z 326 (loss of water molecule from m/z 344), m/z 278 (loss of C6H7O from m/z 386), m/z 269 (by migration of benzyl group to another benzyl group [14] followed by loss of C2H5NO2 from m/z 344), m/z 236 (loss of C7H8O from m/z 344), m/z 235 (loss of H2S from m/z 269) m/z 207 (loss of CO from m/z 235), m/z 179 (loss of C2H3NO from m/z 236), m/z 145 (loss of H2S from m/z 179), m/z 117 (loss of CO from m/z 145), m/z 91 (loss of C3H2O from m/z 145), m/z 65 (loss of C2H2 from m/z 91) and m/z 76 (2-aminoacetic acid moiety). The elemental compositions of all these fragment ions (Scheme S1, Supporting information) have been confirmed by accurate mass measurements and are given in Table S1, Supporting information.

3.3.2. Characterization of DPs

Initially, LC-ESI–MS/MS analysis was tried in both positive and negative modes. All the degradants showed intense [M+H]+ peaks in positive ion mode and very low intensity peaks in negative mode. Thus, the analysis was carried out in positive ionization mode and the MS/MS spectra of the degradation products are shown in Fig. 2. Most plausible structures have been proposed for all the DPs based on the m/z values of their [M+H]+ ions and the MS/MS data in combination with elemental compositions derived from accurate mass measurements. Comprehensive characterization of all the DPs is discussed below.

The LC-ESI–MS/MS spectrum of [M+H]+ of DP 2 (m/z 254, C12H16NO3S+, Rt 2.15 min) shows the product ions at m/z 236 (loss of water), m/z 179 (loss of C2H3NO from m/z 236), m/z 145 (loss ofH2S from m/z 179), m/z 117 (loss of CO from m/z 145), m/z 91 (loss of C3H2O from m/z 145), m/z 65 (loss of C2H2 from m/z 91) and m/z 76. All these fragment ions are structure indicative and highly compatible with the structure, 2-(2-benzyl-3-mercaptopropanamido) aceticacid (Scheme S2, Supporting information). The formation of DP2 under base and acid hydrolytic conditions can be explained by hydrolysis of ester and thio ester bonds to form carboxylic acid and thiol groups (Scheme S4a,b, Supporting information).

The degradation product DP 3 at m/z 296 [M+H]+; C14H18NO4S+ was eluted at 2.46 min. It can be formed by hydrolysis of ester group to form carboxylic acid (Scheme S4a,b, Supporting information). Its MS/MS spectrum shows product ions at m/z 254 (loss of ketene, CH2CO from m/z 296), m/z 236 (loss of water molecule from m/z 254), m/z 208 (loss of CO from m/z 236), m/z 179 (loss of C2H3NO from m/z 236), m/z 145 (loss of H2S from m/z 179), m/z 117 (loss of CO from m/z 145), m/z 91 (loss of C3H2O from m/z 145) and an intense base peak at m/z 76. The formation of an intense peak at m/z 76 indicates that 2-aminoacetic acid moiety of the drug is intact in the DP. The observed fragmentation of protonated DP 3, supported by accurate mass measurements (Table S1, Supporting information), is found to be highly consistent with the proposed structure 2-(3-(acetylthio)-2-benzylpropanamido) acetic acid (Scheme S2, Supporting information).

The DP 4 at m/z 344 ([M+H]+, C19H22NO3S+), formed by hydrolysis of thio ester group (Scheme S4a,b Supporting information), was eluted at 6.20 min. Its MS/MS spectrum shows product ions at m/z 269 (by migration of benzyl group on to another benzyl group [14] followed by loss of C2H5NO2 from m/z 344), m/z 236 (loss of C7H8O from m/z 344), m/z 235 (loss of H2S from m/z 269), m/z 207 (loss of CO from m/z 235), m/z 179 (loss of C2H3NO from m/z 236), m/z 145 (loss of H2S from m/z 179), m/z 117 (loss of CO from m/z 145), m/z 91 (loss of C3H2O from m/z 145), m/z 65 (loss of C2H2 from m/z 91) and m/z 76 (2-aminoacetic acid moiety). Based on these data combined with accurate mass measurements (Table S1, Supporting information), DP 4 was identified as benzyl 2-(2-benzyl-3-mercaptopropanamido) acetate (Scheme S1, Supporting information).

The ESI–MS/MS spectrum (Fig. 2) of protonated DP 5 (Rt 3.10, m/z 268) shows the product ions at m/z 250 (loss of H2O), m/z 236 (loss of methanol from m/z 268), m/z 208 (loss of CO from m/z 236), m/z 179 (loss of C2H3NO from m/z 236), m/z 145 (loss of H2S from m/z 179), m/z 117 (loss of CO from m/z 145), m/z 91 (loss of C3H2O from m/z 145), and m/z 65 (loss of C2H2 from m/z 91). The formation of an intense ion at m/z 90 indicates the presence of 2-aminoacetic acid methyl ester moiety in the degradation product. From the fragmentation studies (Scheme S2, Supporting information), DP 5 was identified as methyl 2-(2-benzyl-3-mercaptopropanamido) acetate. The elemental compositions of [M+H]+ of DP 5 and its fragment ions have been confirmed by accurate mass measurements and are given in Table S1, Supporting information.

The MS/MS spectrum of [M+H]+ of DP 6 (Rt 3.65 min, m/z 310, C15H20NO4S+) is given in Fig. 2. It shows product ions at m/z 278 (loss of methanol moiety from m/z 310, indicating that (2-(3-(acetylthio)-2-benzylpropanamido) ethylidyne) oxonium moiety is intact). The ion at m/z 268 (loss of CH2CO from m/z 310), indicates the presence of methyl 2-(2-benzyl-3-mercaptopropanamido) acetate moiety, m/z 236 (loss of methanol from m/z 268), m/z 221 (loss of C2H3NO from m/z 278), 208 (loss of CO from m/z 236), m/z 179 (loss of C2H3NO from m/z 236), m/z 145 (loss of H2S from m/z 179), m/z 117 (loss of CO from m/z 145), m/z 91 (loss of C3H2O from m/z 145). Based on MS/MS experiments and accurate mass measurements (Table S1, Supporting information), the structure of DP 6 can be assigned as methyl 2-(3-(acetylthio)-2-benzylpropanamido) acetate (Scheme S2, Supporting information).

The ESI–MS spectrum of DP 7 (Rt 4.40 min) shows the [M+H]+ ion peak at m/z 402 with an elemental formula, C21H24NO5S. A mass difference of 16 Da indicates that degradant was formed by an addition of one oxygen atom. MS/MS spectrum of protonated DP 7 displayed product ions at m/z 384 (loss of water molecule), m/z 360 (loss of ketene), m/z 344 (loss of water from m/z 360), and m/z 296 (loss of C7H6O from m/z 402). The ion at m/z 296 can be formed through a McLafferty rearrangement involving the hydroxyl hydrogen at the benzylic carbon and the carbonyl group as shown in the Scheme S3, Supporting information. This also supports the hydroxylation of benzylic carbon under oxidative conditions to form DP 7. It also shows the fragment ions at m/z 285 (formed from m/z 360 by migration of benzyl group on to another benzyl group [14] followed by loss of C2H5NO2), m/z 251(loss of H2S from m/z 285), m/z 149 (from m/z 360), m/z 107 (loss of CH2CO from m/z 149), m/z 91 (from m/z 342), m/z 76 (loss of C19H18O3S from m/z 402) and m/z 65 (loss of C2H2 from m/z 91). From MS/MS data and accurate mass measurements (Table S1, Supporting information), the structure of DP 7 can be assigned as benzyl 2-(2-(acetylthiomethyl)-3-hydroxy-3-phenylpropanamido) acetate.

The isolated DP 1 (Rt 13.65 min, S9 Supporting information) was analyzed by GC-EIMS. The EI spectrum of the sample matched with that of benzyl alcohol from NIST library (Fig. 5). This DP can be easily formed from the drug under hydrolytic conditions as shown in Scheme S4 a,b, Supporting information.

3.4. NMR structural characterization of major degradation products (DP 2 and DP 5)

1H NMR chemical shifts (δ), and 13C NMR chemical shift values are presented for DP 5 and RACE in Table 2. NMR studies of DP 5 reveals that it has 17 hydrogens and 13 carbons. Absence of singlet at δ 2.32 ppm (3H), and presence of triplet at δ 1.66 ppm (SH) confirms the hydrolysis of thioester group. This is also evidenced by absence of a peak at δ 195.93 (Cdouble bond; length as m-dashO) in 13C NMR spectrum of DP 5. 1H and 13C NMR spectra of DP 5 also show the absence

of proton and carbon peaks related to benzyl group and presence of a methyl group at δ 3.73 ppm. DEPT135 and 13C NMR experiments verified that DP 5 contains 1-CH3 (primary), 3-CH2 (secondary), 6-CH (tertiary) and 3-C (quaternary) carbons. DEPT135 spectrum of DP 5 shows presence of new peak at δ 52.28 (18CH3) and absence of peaks related to 20, 21, 23, 24, 25, 26 and 27 carbon atoms which were earlier seen in DEPT135 spectra of RACE. The single bond carbon proton heteronuclear correlation experiments (HSQC) are shown in Fig. 4. HSQC spectrum of DP 5 shows the absence of correlations of CH3 (20), CH2 (21) and CH (23, 24, 25, 26, 27) which were observed in RACE HSQC spectrum. The observations from 1H, 13C, DEPT135 and HSQC NMR spectra confirm that DP 5 is methyl 2-(2-benzyl-3-mercaptopropanamido) acetate (Fig. 3).

The structure of DP 2 was also confirmed by 1H NMR experiments. 1H NMR (SI) values in CDCl3, δ (ppm) 10.32 (s, 1H) 7.39–7.257 (m, 6H, 5 are of aromatic protons and one is CDCl3 proton), 6.06 (s, 1H), 4.20–4.15 (dd, 1H), 3.99–3.95 (dd, 1H), 3.08–3.04 (m, 1H), 2.97–2.94 (m, 1H), 2.71–2.69 (m, 1H), 2.67–2.65 (m, 1H) and 1.771.74 (t, 1H). The 1H NMR spectrum of DP 2 and DP 5 were compared for characterization. The most characteristic peak at δ 10.32 (s, 1H) of carboxylic acid and loss of a peak at δ 3.73 (s, 3H) of methyl group confirms the structure of DP 2 as 2-(2-benzyl-3-mercaptopropanamido) acetic acid.

3.5. Cytotoxicity assay

The toxicity of degradation product, DP 1(benzyl alcohol) was extensively studied and reported in the literature [15], [16], [17], [18], [19], [20], [21] and [22]. Hence, cytotoxicity tests for the isolated degradation products DP 2 and DP 5 were evaluated in the present study. From absorbance values, percentage inhibition was calculated from equation (% Inhibition = (1 − ASample/AControl) × 100), and IC50 values were calculated from concentration vs percentage inhibition graph (GraphPad Prism software). The results indicated (Fig. 6) that the compound DP 5 has significant cytotoxicity (on A549 and Hep G2 cells), whereas DP 2 is nontoxic. The IC50 values of DP 5 on A549 and Hep G2 cells were found to be 58.42 μM (±7.15), 82.6 μM (±9.01), respectively.

4. Conclusion

Forced degradation studies on racecadotril were performed as per ICH guidelines. A total of seven DPs were identified and characterized using LC–MS/MS and GC–MS. This is a typical case of degradation where co solvent methanol reacts with RACE leading to the formation of pseudo DPs, DP 6 and DP 5. Major DPs (DP 2 and DP 5) were isolated and their structures were confirmed by MS/MS and NMR. The cytotoxic activity study of these two DPs revealed that DP2 to be non-toxic whereas DP 5 has potential pulmonary and hepatic toxicity.

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