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Short communication

Isolation and characterization of benazepril unknown impurity by chromatographic and spectroscopic methods

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Abstract

The presence of unknown impurity of the order of 0.2% was identified in benazepril using liquid chromatographic technique employing binary gradient system comprising acetic acid and ammonia in water and acetonitrile as the mobile phase. LCMS data corresponds to hydroxylated benazepril (OHB) derivative possessing the molecular formula $C_{24}H_{28}N_2O_6$. This impurity was isolated using isocratic system containing ammonium acetate and acetonitrile and the product was characterized using FT-IR, ^{1}H and ^{13}C NMR and mass spectroscopy to ascertain the structure of the impurity. The spectroscopic analysis revealed the presence of hydroxyl function on C^{17} carbon atom of benazepril molecule. The plausible mechanism for the formation of OHB species is proposed.

Keywords: Benazepril; Angiotensin converting enzyme inhibitor; Chromatography; LC–MS/MS; NMR; FT-IR; Method development

1. Introduction

Benazepril HCl, {(3S)-3-[(1*S*)-1-ethoxycarbonyl-3-phenyl propyl amino]-2,3,4,5-tetrahydro-2-oxo-1H-1-benzazepin-1-yl acetic acid hydrochloride} [1] is a potent angiotensin converting enzyme inhibitor and it has been used in the treatment of hypertension and congestive heart failure [2–5]. Actually, the hydrolyzed product of benazepril namely benazeprilate, is the potential compound which has been shown to be effective against these disorders [6,7]. So far, six impurities have been found to be associated with benazepril that include benazepin acid, diacid, *S*-amine (starting material), *SR*-diastereomer, cyclohexyl analogue and ethyl ester [8]. We observed the presence of an additional impurity OHB along with the existing ones employing the binary gradient system for the analysis of benazepril. Previously, it was suggested that this impurity could be a hydroxylated benazepril, which might have been generated due to the presence of small amount of moisture (Fig. 1). Based on area normalization method of LC chromatogram, it was found to be about 0.2%. The present communication describes a method development for identification, isolation and characterization of this impurity using spectroscopic techniques.

2. Experimental

2.1. Chemicals

LC-grade water (resistivity less than 18.2 MΩ cm at 25 °C and total organic carbon content less than 5 μg l^{-1}) was prepared by purifying distilled water with a Milli-Q water purification system from Millipore (Bedford, USA). Acetonitrile (gradient grade for chromatography) was purchased from Qualigens (Glaxo SmithKline Pharmaceuticals, Mumbai, India) while acetic acid (∼99% p.a.) was obtained from RanKem (New Delhi, India). AR grade ammonia solution (∼25%) and ammonium dihydrogen phosphates were procured from S.D. Fine Chem. Ltd. (Mumbai, India).

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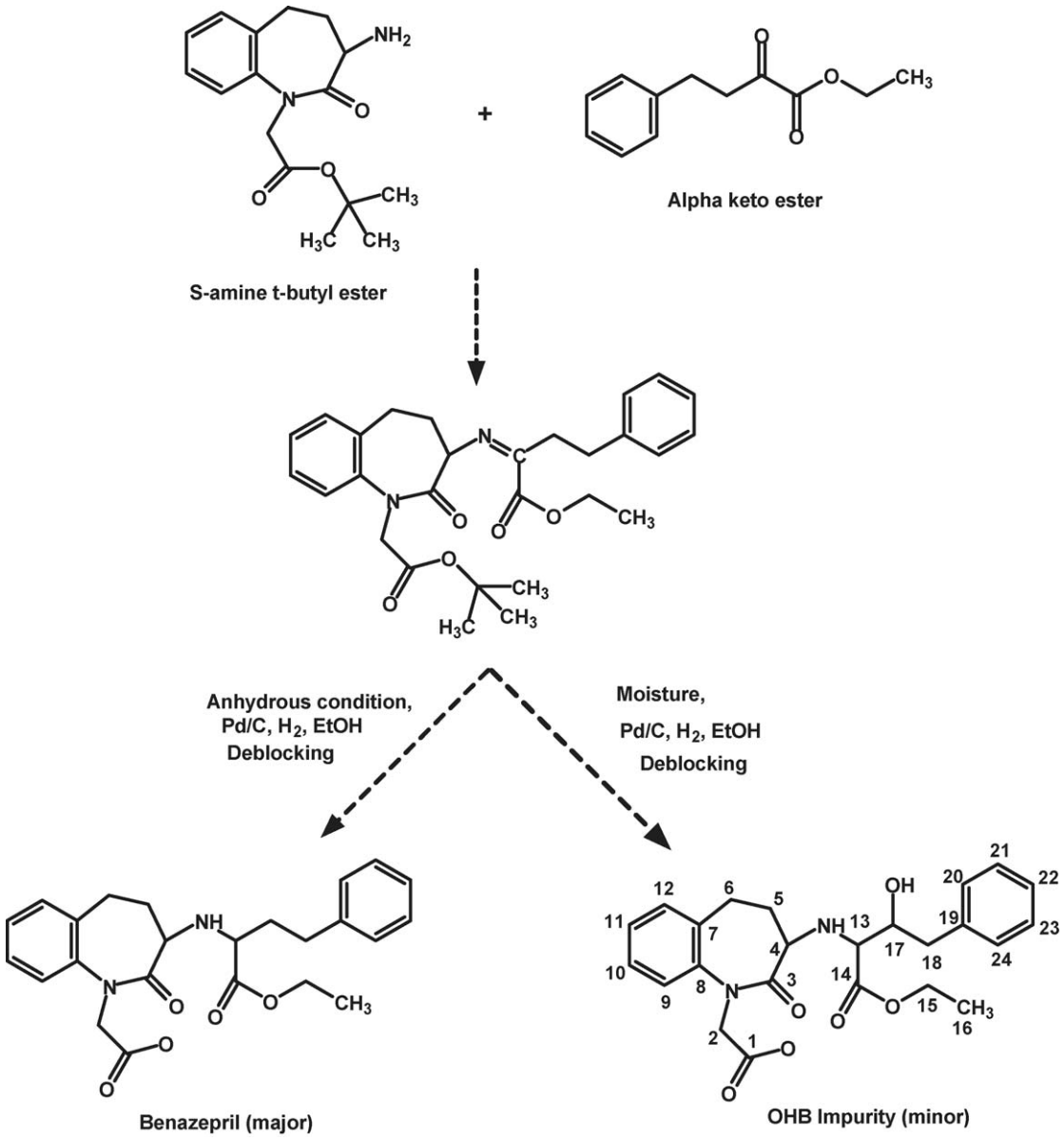

Fig. 1. Proposed formation pathway for benazepril OHB impurity.

2.2. Instrumentation

The LC–UV analysis was performed on Shimadzu LC2010A system. The UV spectra of all peaks were recorded at 240 nm while LC–MS/MS analysis was carried out on Perkin-Elmer liquid chromatograph coupled with a PE SCIEX model API 3000 Triple Quadruple mass spectrometer. A turbo ion spray source (ESI mode) was used for the work described here. The positive ion spray mode was used for samples. MS and MS/MS spectra were obtained under the following conditions: ionization volt energy (IVE), 4000 V at 350 °C, declustering potential 6 V and focusing potential 60 V. For recording CAD spectrum CAD gas (N_2) was set at 6 V and collision energy was set at 29 eV. The isolation of impurity was carried out using preparative HPLC (Shimadzu LC-8A) instrument equipped with a system controller SCL-10 AVP and SPD-10 AVP UV detector and HPLC data was processed using class VP 5.03 software. The ^{1}H and ^{13}C NMR spectra were recorded on 300 MHz Varian machine while FT-IR measurements were performed on Perkin-Elmer FTIR model 1600.

2.3. Preparation of benazepril substance solution for isolation of impurity

Approximately 0.6 g of benazepril was dissolved in 7 ml of distilled water and 1 ml of methanol and the resulting solution was sonicated.

3. Results and discussion

3.1. Method development for impurity identification and isolation

3.1.1. Method development for impurity identification using LC–UV and LC–MS/MS

Suitable methods were developed for identification of unknown impurity using LC–UV and LC–MS/MS. Zorbax RX C-8 column (Agilent, New Port, USA) having 250 mm × 4.6 and 5 μ-particle size was employed for LC–UV. The mobile phase was introduced at the flow rate of 1 ml min^{-1} and injection volume of 20 μL having sample concentration of 1 mg ml^{-1}. The mobile phase (A) was prepared by dissolving 1.04 g of ammonium dihydrogen phosphate in 900 ml distilled water to which 100 ml methanol was added and the pH of the solution was adjusted to 5.0 with phosphoric acid, while HPLC grade methanol was used as another mobile phase (B). These solutions were filtered and degassed separately before the injection. The binary gradient conditions for LC–UV scans were as follows: (a) linear gradient from 95 to 60% of (A) for 0–10 min; (b) linear gradient of 15% of (A) for 10–60 min; (c) the gradient was changed to 95% of (A) for 2 min and (d) reconditioning of the column for 8 min. The gradient response corresponded to benazepril along with its associated impurity that was observed at 24.94 min (Fig. 2). For the verification of the presence of the impurity, already known impurities of benazepril such as benazepin acid, diacid, *S*-amine (starting material), *SR*-diastereomer, cyclohexyl analogue and ethyl ester were spiked and it was observed that this impurity does not correspond to any one of those known impurities. Moreover, it is significant to note that the method developed for the identification of this impurity was most appropriate and gave us better resolutions on the gradient condition employed for its identification. Such an analysis has also provided us the quantitative result for six individual impurities, which was further confirmed by LC–MS data. These investigations thus motivated us to undertake the identification and characterization of OHB using LC coupled with a triple quadruple mass spectrometer.

Since the mobile phase employed for LC–UV analysis of benazepril consisted of non-volatile ammonium dihydrogen phosphates; it was necessary to modify the mobile phase suitable for LC–MS analysis. It was reported earlier that a mobile phase containing small amount of formic acid, acetic acid or trifluro acetic acid could be used as a volatile buffer solution [9]. During our analysis, acetic acid was found to be appropriate for obtaining better results and therefore, it was employed for LC–MS analysis (Fig. 3a).

LC–MS analysis of benazepril sample was carried out using Zorbax XBD C-18 column (250 mm × 4.6 mm, Agilent, New port, USA) having particle size of 5 μm and the mobile phase flow rate was maintained at 1.5 ml min^{-1} with 20 μL injection volume. The benazepril sample concentration was 1 mg ml^{-1} dissolved in mobile phase (D). The column oven temperature was set at 40 °C. The mobile phase (C) contained 0.05% glacial acetic acid in distilled water and the pH of solution was adjusted to 4 with ammonia while mobile phase (D) consisted of mobile phase C and acetonitrile in 20:80 proportion.

The gradient conditions for LC–MS analysis of benazepril sample were from 0 to 7 min, 100% of mobile phase (C); from 7 to 15 min, linear gradient up to 60% of mobile phase (C); from 15 to 25 min, linear gradient up to 50% of mobile phase (C); from 25 to 30 min, linear gradient up to 15% of mobile phase (C); from 30 to 35 min, linear gradient up to 0% of mobile phase (C); from 35 to 36 min, 100% of mobile phase (C) and finally reconditioning column for 9 min on starting conditions.

3.1.2. Method development for impurity isolation using preparative LC

In order to isolate the impurity from benazepril preparation, the isolation conditions for preparative LC were suitably modified so as to suit the column conditions. The isolation was carried out using X-Terra RP18e column (300 mm × 19 mm, 7 μm, Waters, USA) with mobile phase comprising 0.05 M ammonium acetate in distilled water and acetonitrile in 70:30 proportions and the flow rate was maintained at 15 ml min^{-1} for 25 min. Approximately 0.6 g of sample was injected on to column every time and this loading was continued until the sufficient amount of OHB was isolated (∼85 injections). The LC–UV analysis indicated that the OHB impurity was eluted around 5 min on preparative column. The fractions containing >90% of OHB were collected, concentrated using rotary evaporator at 35 °C under vacuum and finally lyophilized to obtain solid product.

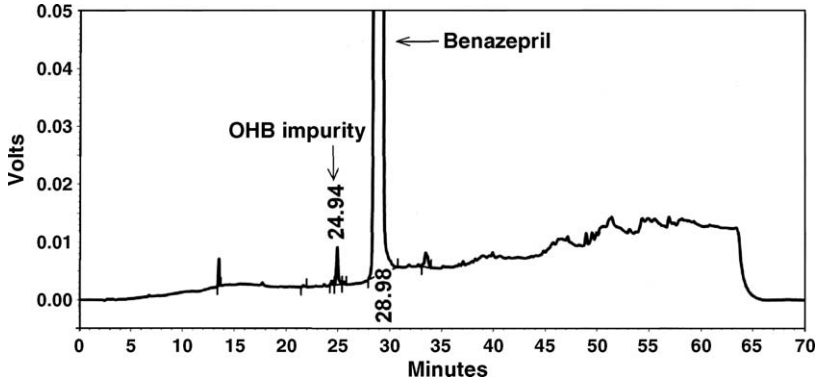

Fig. 2. LC–UV chromatogram of benazepril containing OHB impurity.

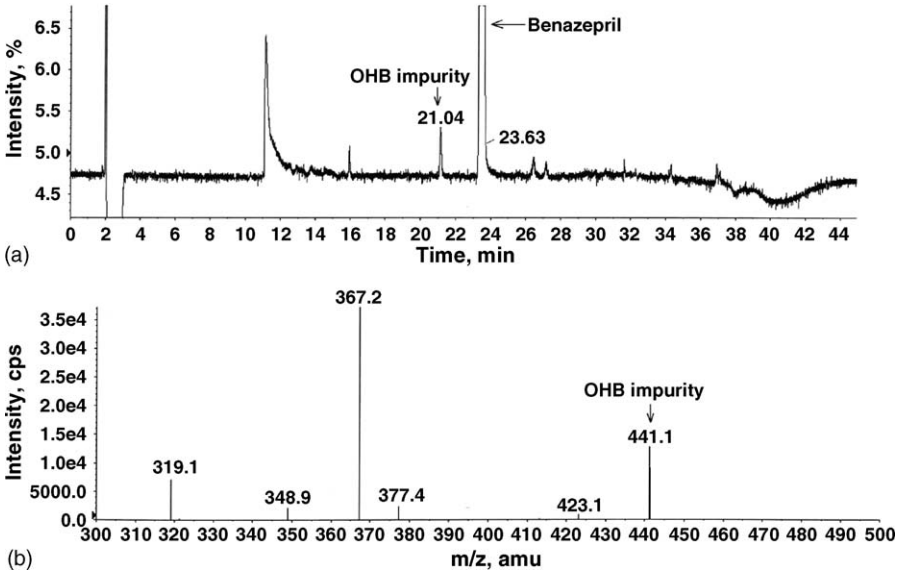

Fig. 3. (a) LC–MS chromatogram of OHB impurity and (b) LC–MS/MS of OHB impurity.

3.2. Characterisation of unknown impurity OHB

The isolated OHB impurity was subjected for structural analysis using LCMS/MS, MS, ^{1}H and ^{13}C NMR and FT-IR spectroscopic methods.

Initially, the molecular mass of impurity was determined from LCMS of benazepril sample which exhibited a peak at *m/z* 441 corresponding to OHB impurity and for the accurate mass measurement; this peak was subjected to further MS analysis (Fig. 3b). It indicated that a peak at 425 corresponds to benazepril while the unknown impurity peak was observed at mass of 441 [(MH^+). Moreover, benazapril and OHB impurity exhibited similar fragmentation pattern suggesting that the impurity degrades in the similar manner [10]. The fragmentation pattern of isolated OHB molecule exhibits seven fragments with *m/z* at 423 ($C_{24}H_{27}N_2O_5$), 377 ($C_{22}H_{21}N_2O_4$), 367 ($C_{21}H_{23}N_2O_4$), 348 ($C_{21}H_{21}N_2O_3$), 319 ($C_{16}H_{19}N_2O_5$), 235 ($C_{12}H_{15}N_2O_3$) and 190 ($C_{12}H_{16}NO$). It is evident from this data that there existed a mass difference of 16 units between benazepril and OHB impurity and it may be explained on the basis of hydroxyl function attached to one of the carbon atoms of the benazepril molecule.

^{1}H NMR spectrum of OHB displays following peaks: δ 7.31–7.13 (m, 9H, aromatic), 4.90 (s, C^{17}–OH), 4.44–4.36 (s, 2H, $C^{2}H_2$), 4.22–4.14 (m, 1H, $C^{17}H$), 4.10–3.96 (m, 2H, $C^{15}H_2$), 3.8–3.76 (d, 1H, C^{13} H), 3.66–3.58 (m, 2H, $C^{4}HNH$), 2.78–2.70 (m, 2H, $C^{18}H_2$), 2.58–2.48 (m, 2H, $C^{6}H_2$), 2.40–2.00 (m, 2H, $C^{5}H_2$) and 1.10 (t, 3H, $C^{16}H_3$). A band at 4.9 δ originating from hydroxyl group of OHB impurity disappeared on D_2O exchange suggesting the substitution –OH at C^{17}. Moreover, the adjacent C^{18} and C^{13} protons exhibited a downfield shift indicating the influence of the hydroxyl function. The splitting patterns of these protons revealed the interactions within the molecule due to three-dimensional orientations as a consequence of the presence of asymmetric carbon centers. It suggested that such geometrical orientation of OHB impurity generates the intra and intermolecular hydrogen bonding contacts thus modulating the spectral behavior. However, remaining peaks for benazepril and OHB impurity were found to be almost identical [11].

^{13}C NMR spectrum of OHB consists of following peaks with their assignments as C^1 (173.0), C^2 (50.0), C^3 (170.8), C^4 (55.5), C^5 (34.5), C^6 (27.5), C^7 (129.2), C^8 (141.5), C^9 (122.5), C^{10} (127.5), C^{11} (125.5), C^{12} (128.9), C^{13} (64.5), C^{14} (172.5), C^{15} (59.6), C^{16} (14.0), C^{17} (72.5), C^{18} (37.0), C^{19} (139.5), C^{20} (128.0), C^{21} (129.2), C^{22} (125.5), C^{23} (129.2) and C^{24} (128.0). The substantial difference was observed for C^{17} resonance, which was shifted towards lower energy side by about 41 ppm indicating the presence of electron withdrawing –OH group. Moreover, the adjacent carbons (C^{18} and C^{13}) also exhibited similar behavior while a minor change was noted for C^4 as a result of its interaction with C^{17} thus corroborating the influence of the hydroxyl group [11,12]. The DEPT 135 carbon analysis indicated the presence of five CH_2 groups instead of six, thus confirming the presence of an OH group on one of the CH_2 group.

The observed IR vibrational modes corresponding to acid –OH and –CH_2 bending stretch revealed differences in terms of nature and peak positions. The former peak appearing at 3455 cm^{-1} in benazepril exhibited downfield shift of about 37 cm^{-1} with while the later band (1524 cm^{-1}) shifted on the lower energy side (1492 cm^{-1}) as a consequence of presence of –OH group on C^{17} [11]. Moreover, the hydroxyl stretch of OHB impurity also contributed for the peak broadening of hydroxyl stretch of acid function suggesting its involvement in hydrogen bonding with carboxylic stretch.

4. Conclusion

From the above study, it was clear that the OHB impurity predicted earlier in the synthesis of benazepril is indeed its hydroxylated derivative and the present work illustrated that this

impurity could be detected in the LC analysis with appropriate designing of the method employed. Moreover, the isolation of this impurity was achieved by tuning the preparative LC method and it was isolated in a sufficient quantity for the first time. The spectroscopic analysis of the isolated impurity indicated that the molecule is hydroxylated derivative as evident from MS/MS, FT-IR and NMR techniques.

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