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LC–UV–PDA and LC–MS studies to characterize degradation products of glimepiride

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ABSTRACT

Degradation products of glimepiride formed under different forced conditions have been characterized through LC–UV–PDA and LC–MS studies. Glimepiride was subjected to forced decomposition under the conditions of hydrolysis, oxidation, dry heat and photolysis, in accordance with the ICH guideline Q1A(R2). The reaction solutions were chromatographed on reversed phase C8 (150 mm × 4.6 mm i.d., 5 μm) analytical column. In total, five degradation products (I–V) were formed under various conditions. The drug degraded to products II and V under acid and neutral hydrolytic conditions while products I, III and IV were formed under the alkaline conditions. The products II and V were also observed on exposure of drug to peroxide. No additional degradation product was shown up under photolytic conditions. All the products, except I, could be characterized through LC–PDA analyses and study of MS fragmentation pattern in both +ESI and −ESI modes. Product I could not be identified, as it did not ionize under MS conditions. The products II, III and V matched, respectively, to impurity B (glimepiride sulfonamide), impurity J and impurity C (glimepiride urethane) listed in European Pharmacopoeia. The product IV was a new degradation product, characterized as [[4-[2-(*N*-carbamoyl)aminoethyl]phenyl]sulfonyl]-3-*trans*-(4-methylcyclohexyl) urea. The degradation pathway of the drug to products II–V is proposed, which is yet unreported.

1. Introduction

Glimepiride is a third generation sulfonylurea type oral hypoglycemic agent, which is widely used in treatment of type 2 diabetes [1,2]. Chemically, it is 1-[[4-[2-(3-ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)-ethyl]phenyl]sulfonyl]-3-*trans*-(4-mehtylcyclohexyl)urea (Fig. 1). The presence of a sulfonylurea bridge, a carboxamide linkage, a constrained lactam ring and an α,β-unsaturated carbonyl system in chemical structure of glimepiride makes the drug susceptible to degradation, due to labiality of these linkages and functional groups to hydrolysis and photolysis [3–10]. As a result, several degradation products are anticipated to be formed during formal stability testing of the drug.

The drug substance monograph of glimepiride in European Pharmacopoeia (EP) lists ten impurities (A–J) [11]. Of the list, four are also mentioned as related substances in the drug monograph by the United States Pharmacopeia [12]. Even some studies on the drug are reported in the literature. Khan et al. [13] developed a LC method for the separation of glimepiride and five related impurities. A more relevant publication is by Kovářıková et al. [14], who carried out HPLC study on glimepiride under hydrolytic (acid, neutral and alkaline) and oxidative stress conditions, but no degradation products were identified.

Thus, the purpose of the present study was to identify the degradation products of the drug formed under ICH recommended stress conditions of hydrolysis, oxidation, dry heat and photolysis [15–17] taking the help of LC–PDA and LC–MS techniques. Another endeavor was to establish pathway for formation of the identified degradation products.

2. Experimental

2.1. Chemicals and reagents

Glimepiride was supplied by Panacea Biotec Ltd. (Lalru, India) as a gift sample. Acetonitrile and methanol (HPLC grade), hydrochloric acid, sodium hydroxide pellets, hydrogen peroxide solution, acetic acid glacial and ammonium acetate (all AR grade) were purchased from Ranbaxy Fine Chemicals (Gurgaon, India). HPLC-grade

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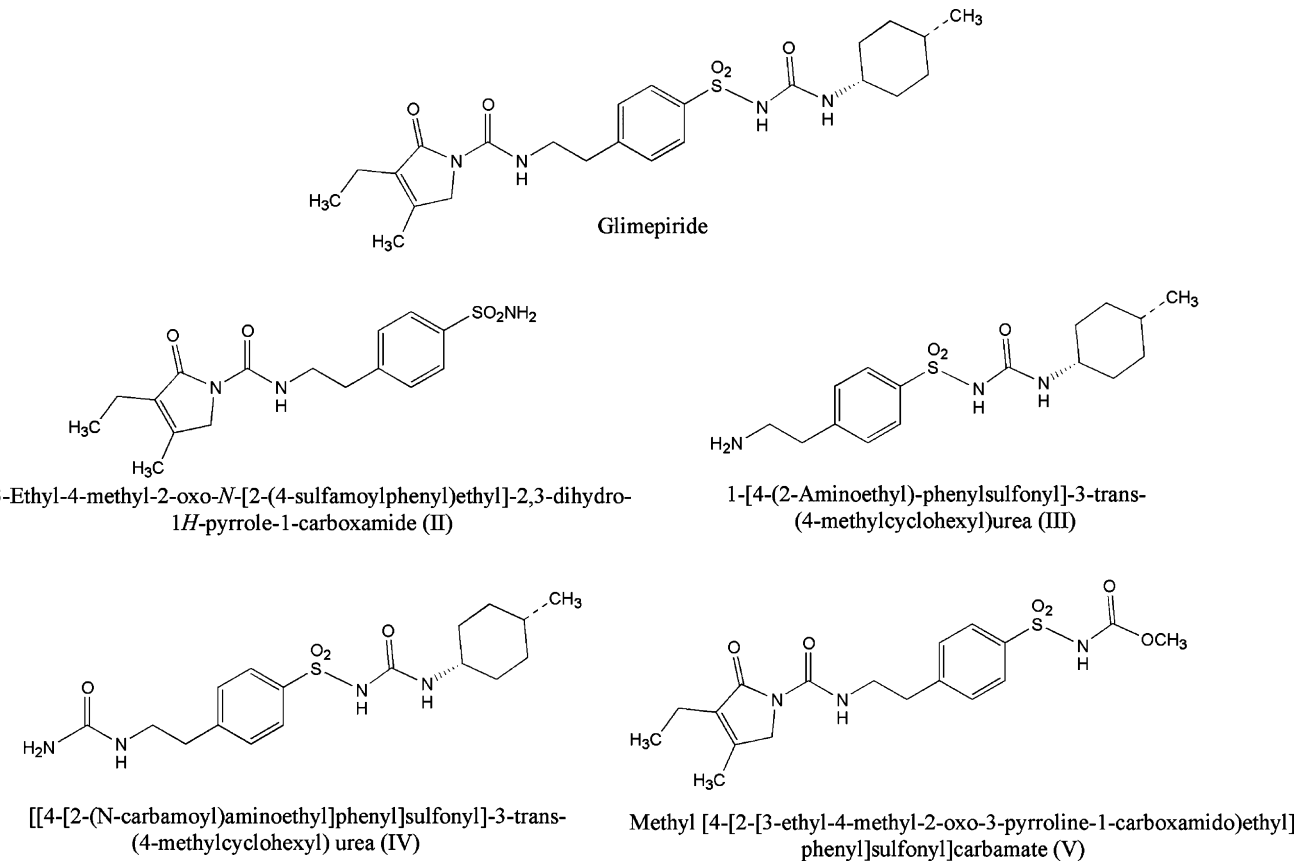

Fig. 1. Glimepiride and its degradation products II–V.

water was produced in the laboratory using a triple-distillation glass assembly (Perfit, Ambala, India). It was filtered through a 0.45 micron membrane (Millipore, Bangalore, India) before use.

2.2. Instrumentation

2.2.1. Forced degradation studies

High precision water bath (Narang Scientific Works, New Delhi, India) capable of controlling the temperature with in ±1 °C was used for generating hydrolytic degradation products. The thermal degradation study was performed using a high precision hot air oven (Narang Scientific Works, New Delhi, India) capable of controlling the temperature with in ±2 °C. Photo-degradation was carried out in a photostability chamber (KBF 240, WTB Binder, Tuttlingen, Germany) equipped with a light bank consisting of two UV (OSRAM L73) and four fluorescent (OSRAM L20) lamps and capable of controlling temperature and humidity in the range of ±2 °C and ±5% RH, respectively. The light system complied with option 2 of the ICH guideline Q1B [18]. At any given time, UV energy and visible illumination were tested using a calibrated radiometer (206, PRC Krochmann GmbH, Berlin, Germany) and a calibrated lux meter (ELM 201, Escorp, New Delhi, India), respectively.

2.2.2. LC–UV analyses

The HPLC system for LC–UV analysis consisted of binary pump (515), dual wavelength detector (2487), Rheodyne manual injector and Millenium 2.01 software (Waters, Milford, USA). The separations were carried out on a Spherisorb® C8 (150 mm × 4.6 mm i.d., 5 μm) analytical column (Waters, Milford, USA) using a mobile phase composed of acetonitrile–ammonium acetate (pH 3.0; 0.02 M) (20:80, v/v) flowing at a rate of 1.0 ml/min. A Nucleosil® C8 (8 mm × 4.6 mm i.d., 5 μm) guard column was placed before the analytical column. The injection volume was 20 μl and eluent was detected at 235 nm. The PDA analysis was performed on HPLC system consisting of a 600 E pump, a 996 photo-diode array (PDA) detector, a 717 autoinjector and a degasser module (Waters, Milford, USA), using the same analytical and guard columns.

2.2.3. MS and LC–MS studies

The MS and LC–MS studies were carried out using positive as well as negative electrospray ionization (+ESI and −ESI) modes on Bruker Daltonics microTOF instrument (Bruker Daltonik GmbH, Bremen, Germany). The data were acquired and processed using microTOF control software ver. 2.0. LC part of the LC–MS comprised of 1100 series system (Agilent Technologies Inc., CA, USA), controlled by Hystar (ver. 3.1) software. The chromatographic conditions used for LC–MS analyses were the same as that for LC–UV analyses, except that injection volume was 10 μl. A splitter was placed before the mass detector, allowing entry of only 35% of the eluent. The operating conditions for MS scan of glimepiride in +ESI mode were optimized as follows: end plate offset, −500 V; capillary, 4500 V; collision cell RF, 500.0 V_{pp}; nebulizer, 1.2 bar; dry gas, 5.0 l/min, and dry temperature, 180 °C. The operating conditions for MS scan of the drug in −ESI mode were same as in +ESI mode, except that nebulizer, dry gas and dry temperature were set at 1.2 bar, 8.0 l/min and 250 °C, respectively. The operating conditions for LC–MS scans of degradation products in −ESI mode were optimized as follows: end plate offset, −500 V; capillary, 4500 V; collision cell RF, 300.0 V_{pp}; nebulizer, 1.2 bar; dry gas, 6.0 l/min, and dry temperature, 180 °C. The same operating conditions were employed for LC–MS scans of the degradation products in +ESI mode, except that collision cell RF was increased to 500.0 V_{pp}. The

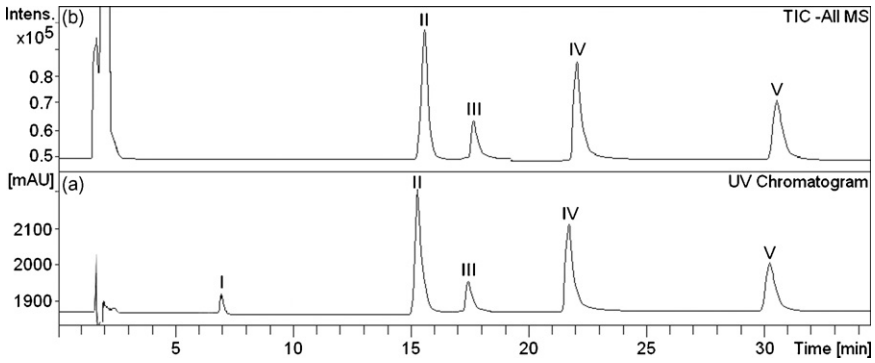

Fig. 2. LC–UV chromatogram (a) and LC–MS chromatogram (b) of the mixture solution containing all the degradation products of glimepiride.

operating conditions for MS^2 scan of drug in both +ESI and −ESI modes were same as those for MS scan of drug in +ESI mode. The same conditions were employed for LC–MS^2 scans of degradation products in −ESI mode but for that in −ESI mode the nebulizer and dry gas were set at 1.2 bar and 6.0 l/min, respectively. The MS scans of the drug and LC–MS scans of all degradation products in both the ionization modes were recorded in the range of 50–3000 *m/z* and masses of the peaks were recorded up to fourth decimal precision for accurate mass measurements.

2.3. Forced degradation studies

The drug was subjected to hydrolytic and photolytic degradation in acidic (0.1N HCl), neutral (water) and alkaline (0.1N NaOH) media and to oxidative degradation in 10% H_2O_2 solution using drug concentration of 0.1% (w/v). The drug solution in acid medium was prepared by dissolving 0.1 g of glimepiride in 55 ml of methanol, adding 0.85 ml of concentrated HCl, and making up the volume to 100 ml with water. The drug solution in water was prepared similarly as above, but without adding HCl. The drug solution in alkaline medium was prepared by dissolving 0.1 g of the drug in sufficient 0.1N NaOH solution to produce 100 ml. The drug solution in oxidative medium was prepared by dissolving 0.1 g of glimepiride in 55 ml of methanol, adding 33 ml of 30% H_2O_2, and making up the volume to 100 ml with water. The hydrolytic degradation products were generated by keeping the drug solutions at 85 °C for 72 h. The same solutions were also subjected to photolytic degradation at 40 °C by exposing to a total dose of 1.2 million lx h of fluorescent and 200 Wh/m^2 of UV-A illumination. A parallel set of the drug solutions was stored in dark at the same temperature to serve as control. The oxidative degradation was carried out at room temperature for 72 h. The dry heat degradation was carried out by exposing the drug, sealed in amber coloured glass vials, to a temperature of 50 °C for 31 days.

2.4. Characterization of degradation products

The stressed solutions, in which sufficient amounts of products were formed, were combined in equal proportions to prepare a mixture containing all the products in one solution. This mixture was subjected to LC–PDA and LC–MS analyses for characterization of the degradation products.

3. Results and discussion

3.1. Stress decomposition behaviour

In total, five degradation products (I–V) were formed under different forced conditions. Two degradation products (II and V) were generated on subjecting the drug to acidic and neutral hydrolysis and oxidation, while other three products (I, III and IV) were formed on alkaline hydrolysis. The behaviour was similar to that reported by Kovářıková et al. [14]. The drug was stable to dry heat as no decrease in area of drug peak was observed during thermal degradation. No photo-degradation product was formed in acidic, neutral and alkaline media, though drug decomposition in all the media was accelerated by light.

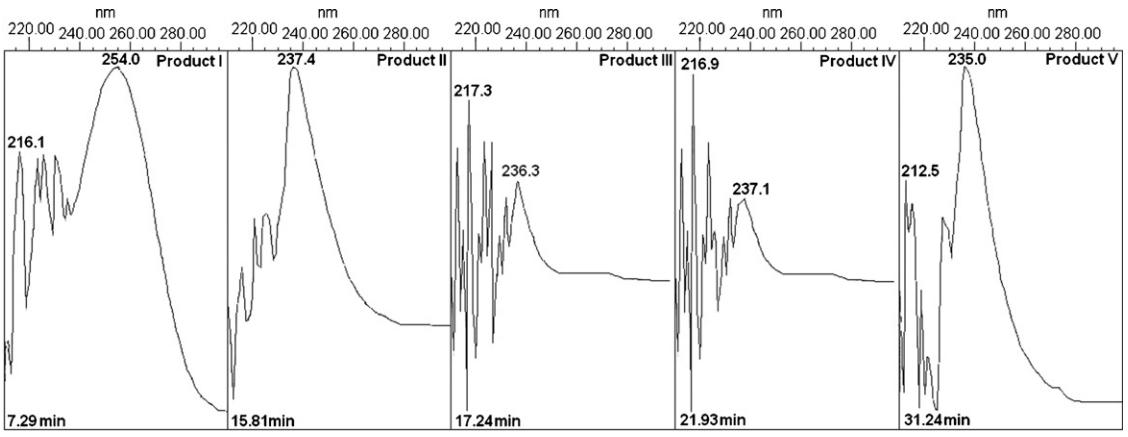

Fig. 3. UV absorbance spectra of degradation products (I–V) of glimepiride.

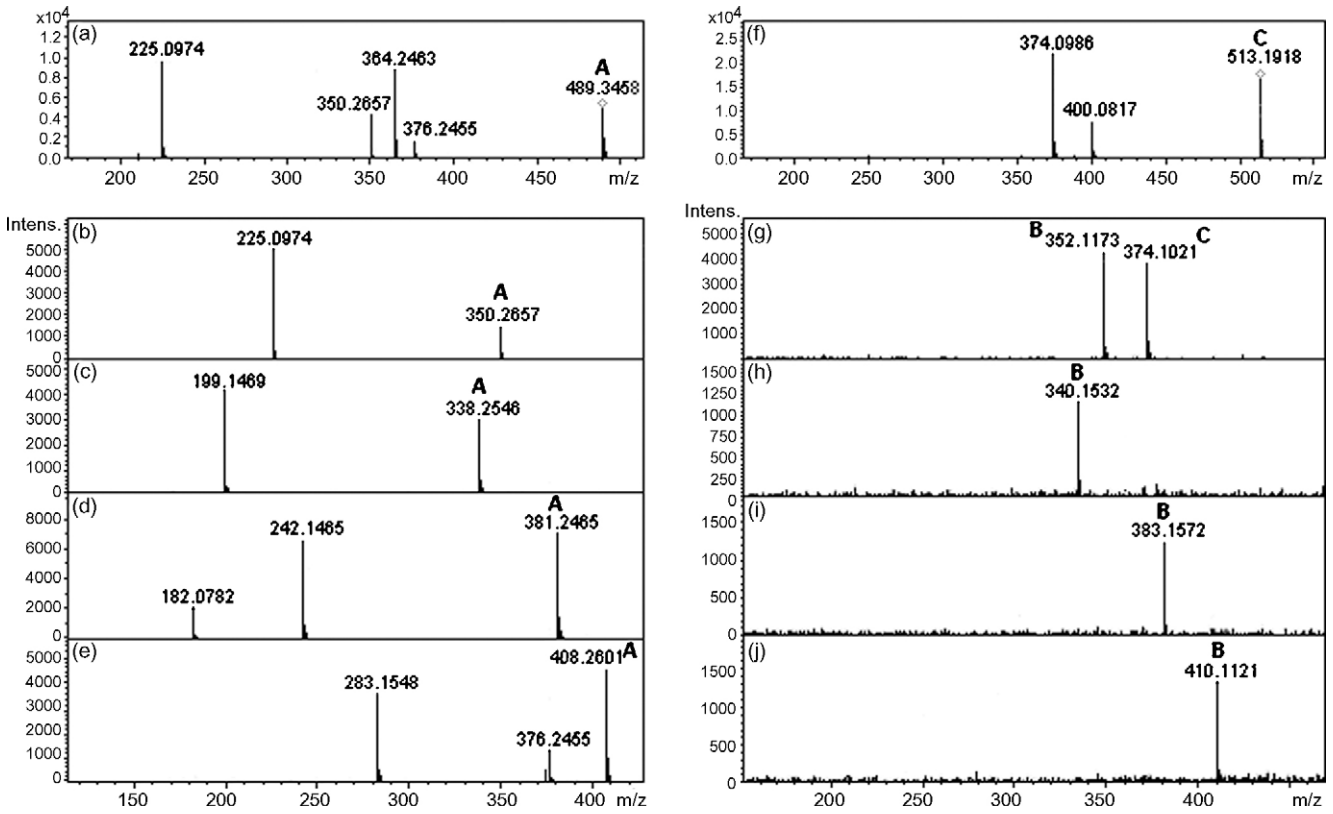

Fig. 4. MS^2 spectrum of glimepiride (a) and LC–MS spectra (b–e) of its degradation products II, III, IV and V, respectively, in −ESI mode, and MS^2 spectrum of glimepiride (f) and LC–MS spectra (g–j) of its degradation products II, III, IV and V, respectively, in +ESI mode. A, B and C refer to peaks due to $[M-H]^-$, $[M-H]^+$ and $[M-Na]^+$ ions, respectively.

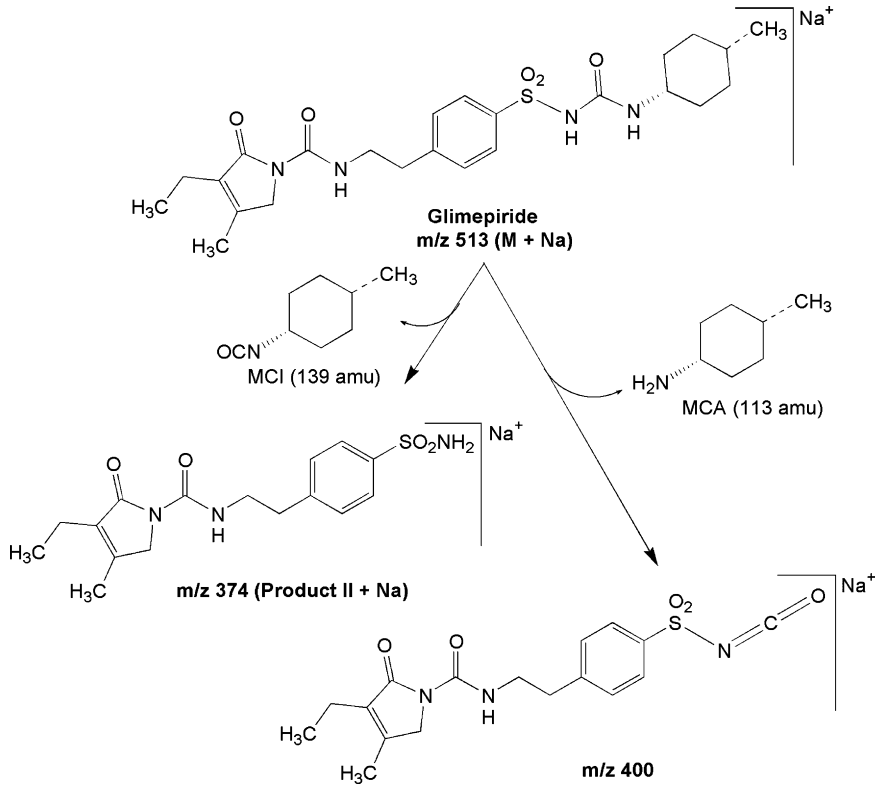

Fig. 5. Mass fragmentation pattern of glimepiride in +ESI mode.

Table 1
Accurate masses of precursor and fragment ions of glimepiride and its degradation products (II–V) in different MS conditions

MS condition	Glimepiride	Degradation product			
		II	III	IV	V
+ESI/MS (precursor ions)	513.1918[a] (513.2148)	374.1021[a] (374.1150) 352.1173[b] (352.1331)	340.1532[b] (340.1695)	383.1572[b] (383.1753)	410.1121[b] (410.1386)
+ESI/MS^2 (fragment ions)	400.0817 (400.0943) 374.0986 (374.1150)				
−ESI/LC–MS					
Precursor ions	489.3458[c] (489.2172)	350.2657[c] (350.1175)	338.2546[c] (338.1538)	381.2465[c] (381.1597)	408.2601[c] (408.1229)
Fragment ions	376.2455 (376.0967) 364.2463 (364.1331) 350.2657 (350.1175) 225.0974 (225.0333)	225.0974 (225.0333)	199.1469 (199.0541)	242.1465 (242.0599)	376.2455 (376.0967) 283.1548 (283.0389)

Masses in parentheses are the actual masses of the ions.
[a] $[M+Na]^+$.
[b] $[M+H]^+$.
[c] $[M-H]^-$.

3.2. LC–UV–PDA study

LC–UV chromatogram showing separation of all the degradation products of glimepiride using a PDA detector is shown in Fig. 2a. The purity angle of each degradation product peak was less than its purity threshold which indicated that all of them were pure. Fig. 3 shows UV absorption spectra of degradation products I–V. It clearly projects that UV absorption behaviour of products II–V was similar with λ_{max} of about 236 nm which matched with λ_{max} of the drug at 235 nm. However, product I had a different absorbance maximum of 254 nm, indicating that it had a different chromophore than the other degradation products. This suggested that the drug chromophore was lost or altered during conversion of glimepiride to product I, while it was unaffected during conversion of drug to the other products.

3.3. MS and LC–MS studies

Glimepiride and its degradation products II–V were detected as $[M-H]^-$, $[M+H]^+$ precursor ions or $[M+Na]^+$ adduct ion at *m/z* values corresponding to more than 99.97% of their actual mass values (Fig. 4 and Table 1). Even the mass values of fragments of each analyte were more than 99.97% of their actual masses (Table 1). The precursor ions of glimepiride corresponded to its molecular mass of 490 amu while molecular masses of products II–V were established to be 351, 339, 382 and 409, respectively.

3.3.1. Mass fragmentation of glimepiride

The base peak at *m/z* 374 in +ESI MS^2 spectrum of the drug was formed by loss of 139 amu from the precursor ion at *m/z* 513 (Fig. 5), similarly as peak at *m/z* 352 is reported to be formed from $[M+H]^+$ precursor ion at *m/z* 491 [19,20]. Formation of these ions by loss of 139 amu may be assigned to elimination of 4-methylcyclohexylisocyanate (MCI) from the respective precursor ions. The other fragment ion at *m/z* 400 formed by loss of 113 amu is proposed to be due to elimination of 4-methylcyclohexylamine (MCA) (Fig. 5). It indicated that even the fragments existed as Na^+ adducts in +ESI mode.

The −ESI MS^2 spectrum of the drug (Fig. 4a) showed four fragment peaks at *m/z* 376 (16%), 364 (92%), 350 (43%) and 225 (100%). The mass fragmentation pattern of glimepiride in −ESI mode is proposed in Fig. 6. Formation of the heaviest fragment ion at *m/z* 376 (F_1) is rationalized by loss of MCA (113 amu) from the precursor ion at *m/z* 489 similarly as in +ESI mode. The fragment of *m/z* 364 (F_2) was formed possibly by loss of 3-ethyl-4-methyl-1,5-dihydropyrrol-2-one of 125 amu from the precursor ion. A difference of 139 amu between *m/z* 489 and fragment ion at *m/z* 350 (F_3) indicated that the latter was formed by loss of MCI from the former (Fig. 6), similarly as *m/z* 352 was formed from adduct ion at *m/z* 513 in +ESI mode (Fig. 5). It was further supported by our earlier reports on mass fragmentation patterns of glipizide [9] and glibenclamide [10]. Formation of *m/z* 225 (F_4) directly from precursor ion by loss of 264 amu did not seem possible. However, appearance of fragment peak at *m/z* 225 in −ESI LC–MS spectrum of product II (Fig. 4b), whose $[M-H]^-$ precursor ion appeared at *m/z* 350, suggested that F_4 was formed by loss of 125 amu from F_3, similarly as F_2 was formed from $[M-H]^-$ precursor ion of the drug. The possible structures of all fragments in the proposed mass fragmentation pattern of glimepiride in −ESI mode suggested that a common fragment (Fc) due to 4-(2-aminoethyl)benzenesulfonyl moiety of the drug was present in all fragments and hence, the drug molecule was visualized as divided into right and left sides attached through the Fc (Fig. 6). Therefore, it can be proposed that mass fragmentation in +ESI mode occurs due to ionization in right side of the drug molecule while ionization in both left and right sides was responsible for fragmentation in −ESI mode.

3.3.2. Identification of degradation products

None of the degradation products could be isolated from the reaction solutions by solvent extraction or crystallization. Hence, the mixture of stressed solutions (refer to Section 2.4) was subjected to LC–MS analyses to characterize the degradation products. As shown in Fig. 2b, the product I was not detected during LC–MS study, possibly due to poor ionizability. Hence it could not be characterized. The degradation products II–V were detected as $[M-H]^-$, $[M+H]^+$ precursor ions or $[M+Na]^+$ adduct ion at *m/z* values corresponding to more than 99.97% of their actual mass values which established their molecular masses to be 351, 339, 382 and 409, respectively (Table 1). The products II–V were characterized (Fig. 1) through comparison of mass fragmentation pattern of glimepiride and degradation products in +ESI and −ESI modes (Figs. 5 and 6).

The molecular mass of product II was found to correspond to molecular mass of impurity B (glimepiride sulfonamide) [11,13]. Further, a fragment peak at *m/z* 225 in −ESI LC–MS spectrum and similar appearance of fragment peaks at *m/z* 350 and *m/z* 225 in −ESI MS^2 spectrum of the drug suggested the product II to be F_3. Hence based on these observations and our earlier reports on mass fragmentation behaviour of sulfonylurea drugs [8–10], the structure of product II was established as 3-ethyl-4-methyl-2-oxo-*N*-[2-(4-sulfamoylphenyl)ethyl]-2,3-dihydro-1H-pyrrole-1-carboxamide.

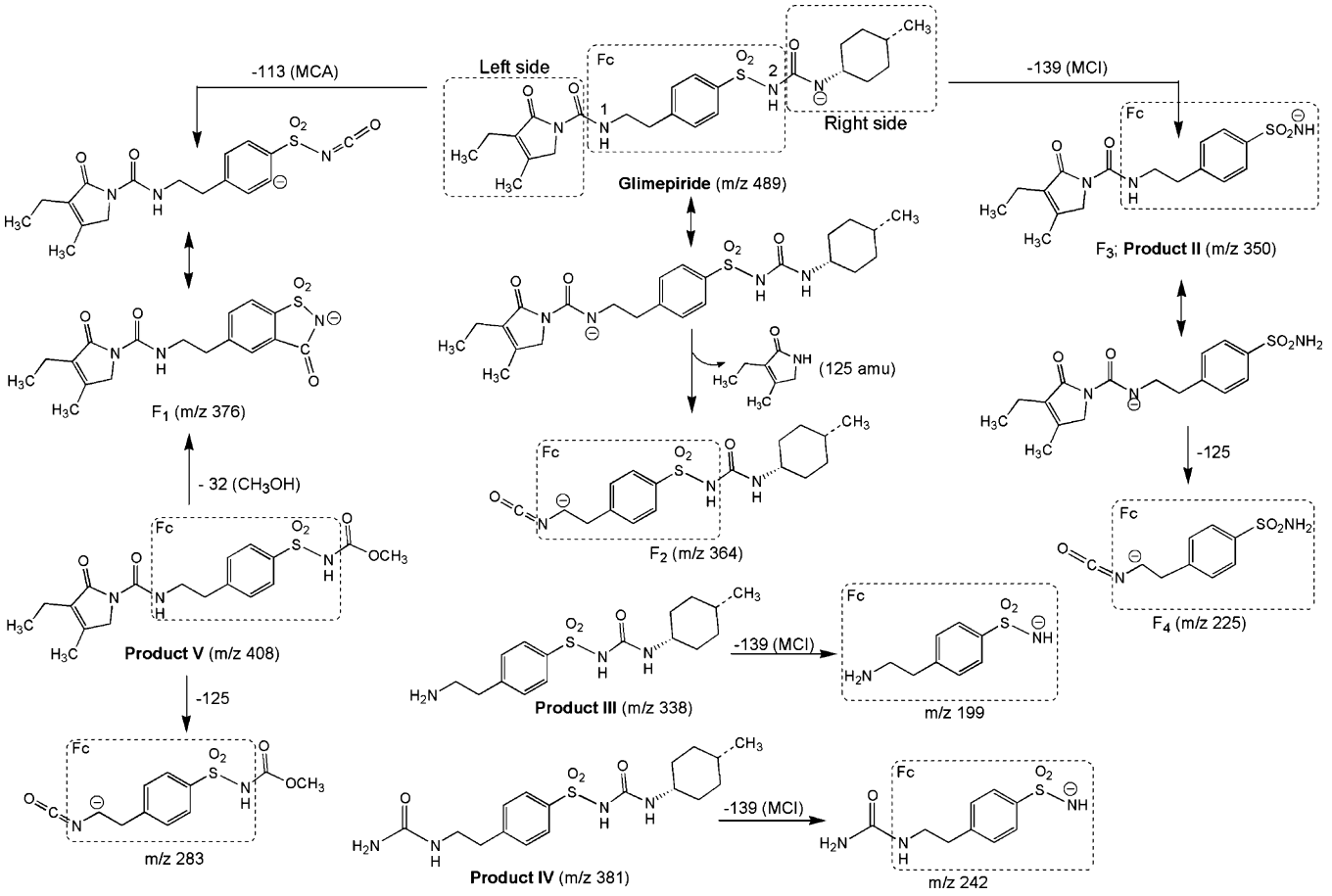

Fig. 6. Proposed mass fragmentation pattern of glimepiride and its degradation products in −ESI mode.

The molecular mass of product III corresponded to molecular mass of impurity J in the monograph on glimepiride in EP [11]. A difference of 139 amu between the lone fragment at *m/z* 199 and the $[M-H]^-$ precursor ion (*m/z* 338) suggested that the former was formed by loss of MCI from the latter, similarly as F_3 was formed from the drug (Fig. 6). It indicated the presence of 4-methylcyclohexylaminocarbonyl moiety in product III. Further, the fragment peak at *m/z* 199, similar to the one reported for glipizide [9] and glibenclamide [10], indicated the presence of 4-(2-aminoethyl)benzenesulfonyl moiety (Fc) in product III. Thus, it revealed that product III was comprised of 4-methylcyclohexylaminocarbonyl and Fc, and hence structure of product III was established as 1-[4-(2-aminoethyl)phenylsulfonyl]-3-*trans*-(4-methylcyclohexyl)urea, matching with impurity J.

The molecular mass of product IV did not match with molecular mass of any known impurity of the drug. A difference of 139 amu between the fragment ion at *m/z* 242 and the $[M-H]^-$ precursor ion (*m/z* 381) suggested that the former was formed by loss of MCI from the precursor ion, similarly as F_3 was formed from the drug, and *m/z* 199 was formed from product III (Fig. 6). It indicated the presence of 4-methylcyclohexylaminocarbonyl moiety (140 amu) in structure of product IV. Mass fragmentation of glimepiride indicated that fragment formed by loss of MCI contained Fc of mass 198 amu. Further, a difference of 44 amu between fragment of *m/z* 242 and Fc of 198 amu suggested that N_1 of Fc was bonded to an entity of mass 44 amu (Fig. 7). This mass of 44 amu could be contributed by N_2O, CO_2, $-CONH_2$, $-NHCH_2CH_3$ or $CH_2{=}CHOH$ [21]. However, retention of Fc (containing two nitrogen atoms) in *m/z* 242 formed by elimination of MCI (containing one nitrogen atom) from the product IV suggested that entity of 44 amu should contain an odd number of nitrogen so that the total number of nitrogen atoms in product IV becomes 'even' to comply with its 'even' molecular weight (Nitrogen Rule). Hence, N_2O, CO_2, and $CH_2{=}CHOH$ were ruled out as probable contributors of 44 amu. Formation of bond between N_1 of Fc and nitrogen of $-NHCH_2CH_3$ under the given alkaline conditions was not supported by any literature report, and consequently $-NHCH_2CH_3$ was also ruled out. In the drug molecule, the N_1 of Fc is bonded to C=O group and hence, based on the probability of $-CONH_2$ contributing mass of 44 amu, the three components,

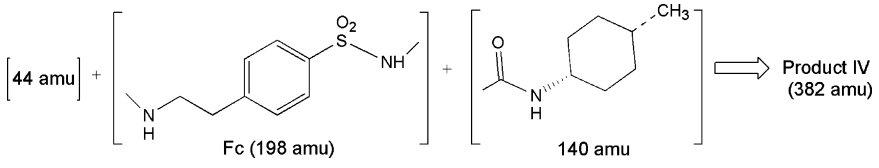

Fig. 7. Assembly of fragments to generate structure of product IV.

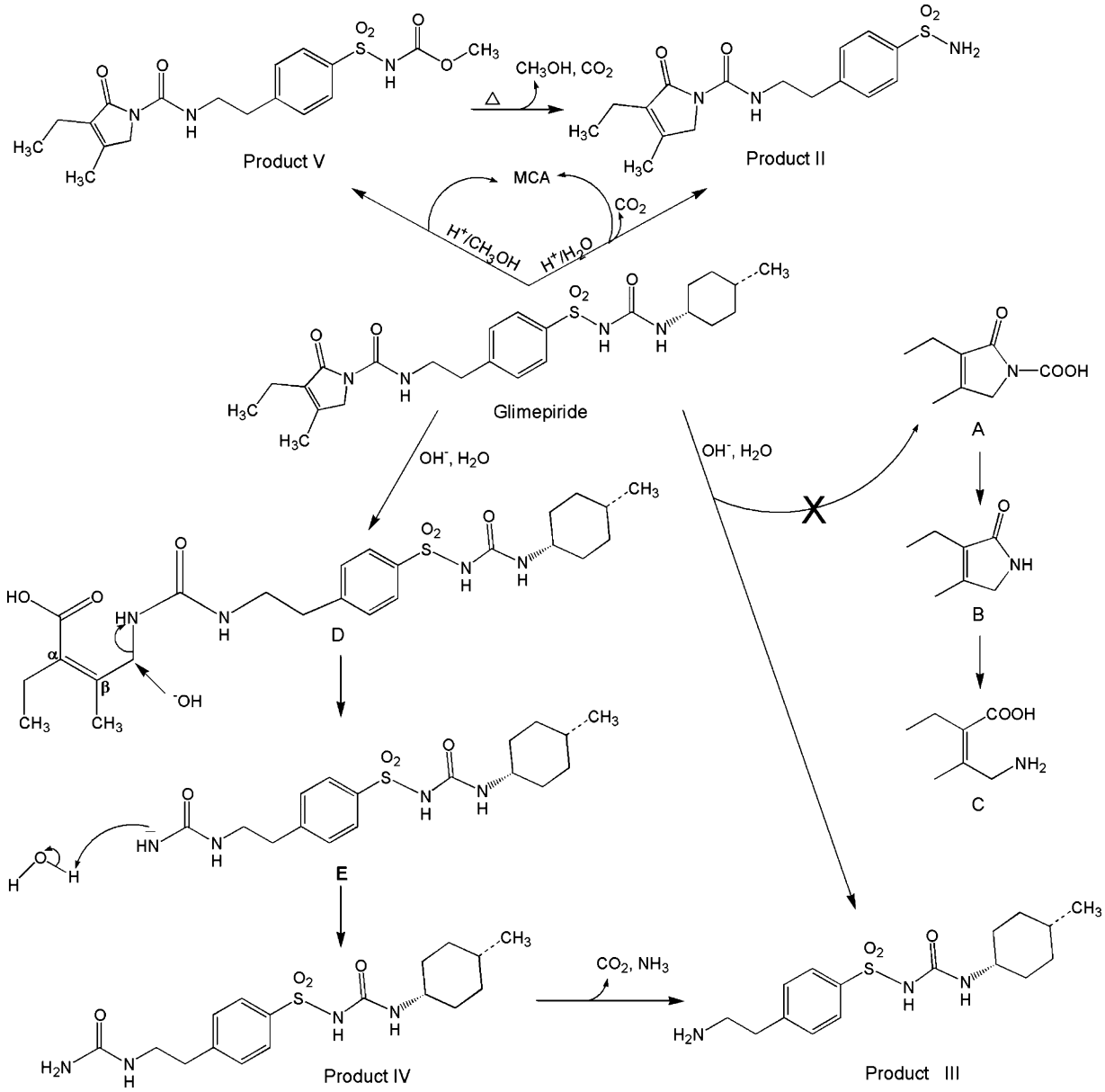

Fig. 8. Pathways of degradation of glimepiride to products II–V.

i.e., $-CONH_2$, Fc (198 amu) and 4-methylcyclohexylamino carbonyl (140 amu) were assembled to generate structure of product IV (Fig. 7) as [[4-[2-(*N*-carbamoyl)aminoethyl]phenyl]sulfonyl]-3-*trans*-(4-methylcyclohexyl) urea which also satisfied the nitrogen rule and its mass fragmentation.

The molecular mass of product V matched with impurity C (glimepiride urethane) in EP [11]. Two fragment peaks at *m*/*z* 376 and *m*/*z* 283 were also noted in the −ESI LC–MS spectrum (Fig. 4e). The fragment at *m*/*z* 376, appearing also in MS^2 spectrum of glimepiride (Fig. 4a), indicated that Fc and left side of the drug molecule were present in structure of product V (Fig. 6). A difference of 32 amu between *m*/*z* 376 (F_1) and the $[M-H]^-$ precursor ion (*m*/*z* 408) could be attributed to loss of CH_3OH, which indicated presence of $-OCH_3$ [22] in product V. The fragment of *m*/*z* 283 was proposed to form by loss of 125 amu (3-ethyl-4-methyl-1,5-dihydropyrrol-2-one) from the precursor ion, similarly as F_4 was formed from F_3, and F_2 was formed from the precursor drug ion (Fig. 6). It further lent support to the presence of $-OCH_3$ group on the right side of structure of product V. Hence, product V was characterized as EP impurity C [11].

3.4. Postulated degradation pathway

The pathway of degradation of glimepiride to the characterized degradation products is outlined in Fig. 8. It is postulated that the drug degraded to sulfonamide product II through hydrolytic cleavage of amide linkage in sulfonylurea moiety. The same is in line with the known hydrolytic behaviour of sulfonylureas in acidic medium [3,9,10], involving either O- or N-protonation, followed by nucleophilic attack of water on the carbonyl carbon [8] resulting in cleavage of C–N bond to produce a carbamic acid intermediate (CAI), which decarboxylated [23] further to produce the sulfonamide product.

The carbamate degradation product V was formed as a result of hydrolysis of the sulfonylurea bridge by attack of more nucleophilic methanolic oxygen on electrophilic C=O carbon of sulfonylurea

bridge. The carbamate was subsequently got hydrolysed to an unstable CAI, which finally decarboxylated [23] to sulfonamide, as described in Fig. 8.

The conversion of glimepiride to product III in alkaline medium occurred due to hydrolysis of the carboxamide linkage, similarly as in glipizide [9] and glibenclamide [10]. However, this route was also expected to generate 3-ethyl-4-methyl-2-oxo-2,5-dihydropyrrole-1-carboxylic acid (A), 3-ethyl-4-methyl-1,5dihydropyrrol-2-one (B) or 4-amino-2-ethyl-3-methyl-but-2-enoic acid (C) (Fig. 8). Incidentally, neither of the products A–C was detected during LC–MS studies, which suggested that product III was not formed by direct hydrolysis of amide linkage connecting Fc with left side of the drug molecule. Formation of product IV from glimepiride in alkaline medium could be explained through reactivity of strained α,β-unsaturated γ-lactam (Fig. 8). The lactam bridge was readily hydrolysed in alkaline medium to generate an intermediate unstable product (D), which was immediately attacked by OH^- ion at electron deficient methylene carbon flanked by –NH– of ureido moiety and sp^2 hybridized β-carbon of α,β-unsaturated carbonyl system to form an intermediate anion (E). The latter abstracted hydrogen from water molecule to generate product IV and regenerated the OH^- ion. Subsequently, the urea moiety of product IV was hydrolysed to product III with elimination of ammonia and carbon dioxide.

The acceleration of decomposition of glimepiride by light to sulfonamide and urethane products could be attributed to photocleavage of N–C(O) bond, in consonant with photolytic degradation of sulfonylureas [24–26]. Similar acceleration of degradation of the drug in alkaline solution by light was also attributed to the photolytic cleavage of the lactam bridge, as reported in an earlier report on glipizide [9].

4. Conclusions

Five degradation products (I–V) were formed during forced degradation study on glimepiride under different conditions. All the products were separated in a single run by an isocratic LC–UV method. The products II, III and V were characterized as known impurities while the product IV was characterized as a new degradation product through mass fragmentation studies. The molecular masses of the degradation products were established by recording LC–MS scans in both +ESI and −ESI modes. The product I was not detected even in both the modes, probably due to its poor ionizability. The products II and V were proposed to form due to hydrolysis of sulfonylurea bridge, whereas, the products III and IV were proposed to form due to hydrolysis of the lactam bridge.

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