Assessment of the Stability of Novel Antibacterial Triazolyl Oxazolidinones Using a Stability-Indicating High-Performance Liquid Chromatography Method

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Key Words

Triazolyl oxazolidinones • Stability • High-performance liquid chromatography • Mass spectrometry

Abstract

Objectives: To evaluate the stability of 12 triazolyl oxazolidinone (TOZ) derivatives in simulated gastric and intestinal fluids as well as in human plasma at $37 \pm 1^\circ\text{C}$. **Materials and Methods:** A stability-indicating high-performance liquid chromatography (HPLC) procedure with a C_8 column (250×40 mm, $5 \mu\text{m}$ particle size) and a mobile phase of acetonitrile/ H_2O (50/50 v/v) at 1.0 ml/min was used. Accelerated stability studies were conducted at $37 \pm 1^\circ\text{C}$ in 0.1 M HCl solution as simulated gastric fluid and in phosphate buffer solution (pH about 7.4) as simulated intestinal fluid. The stability of TOZs in human plasma at a simulated biological temperature of $37 \pm 1^\circ\text{C}$ was evaluated as well. **Results:** The stability studies indicated that the examined TOZs were stable in the above media, with the exception of compounds 1a [tert-butyl 4-(4-((R)-5-((1H-1,2,3-triazol-1-yl)methyl)-2-oxooxazolidin-3-yl)-2-fluorophenyl)piperazine-1-carboxylate] and 1b [tert-butyl 4-(2-fluoro-4-((R)-5-((4-methyl-1H-1,2,3-triazol-1-yl)methyl)-2-oxooxazolidin-3-yl)phenyl) piperazine-1-carboxylate], which underwent degradation in simulated gas-

tric fluid. The degradation kinetics revealed degradation parameters (k_{deg} , $t_{1/2}$, t_{90}) of 0.180 h^{-1} , 3.85 h, and 0.58 h for 1a and of 0.184 h^{-1} , 3.76 h and 0.57 h for 1b, respectively. Furthermore, the degradation products were identified by mass-spectrometric analysis at mass-to-charge ratios 347.5 and 361.5, respectively, and proton nuclear magnetic resonance analysis. **Conclusion:** With the exception of compounds 1a and 1b, the TOZs are stable in simulated gastric and intestinal fluids as well as in human plasma. Being carbamate derivatives, compounds 1a and 1b underwent fast and complete degradation in simulated gastric fluid. The obtained results should be considered for future studies of formulation of structurally related TOZs in oral dosage forms.

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Introduction

The oxazolidinones represent a novel class of antibacterial agents with potent activity against susceptible and resistant Gram-positive bacterial pathogens. Linezolid (fig. 1), the only member of this class in clinical use, has demonstrated significant success in the treatment of a variety of infections caused by Gram-positive bacteria, including hospital and community-acquired pneumonia,

Fig. 1. Chemical structures of antibacterial oxazolidinones.

and skin infections [1–4]. In addition, linezolid showed in vitro activity against selected Gram-negative pathogens, including *Moraxella catarrhalis* and *Haemophilus influenzae*. Oxazolidinone antibacterials inhibit protein synthesis through binding to the bacterial 23S ribosomal RNA of the 50S subunit [5, 6]. Most recently, the crystal structure study by Duffy et al. [7] showed that linezolid binds to the 50S A-site. Moreover, recent reports of linezolid-resistant strains emphasized a pressing need for more effective derivatives with reduced toxic side effects [8–10].

Our previous reports [11–14] and those of others [15, 16] have shown that the replacement of the 5-acetamido methyl substituents of linezolid with the N-linked triazolyl moiety gave 5-triazolylmethyl oxazolidinone derivatives (PH-027, 1–3; fig. 1) with potent antibacterial activity. Furthermore, recent studies by Reck et al. [15] and Hauck et al. [16] reported that a series of novel oxazolidinones bearing different 4'-substituted 1,2,3-triazoles at the C5 position of the oxazolidinone (2–3; fig. 1) showed potent antibacterial activity with diminished monoamine oxidase A inhibitory activity. However, selected novel triazolyl oxazolidinones (TOZs) from our laboratory, with potent in vitro antibacterial activity superior or comparable to linezolid, showed 4-fold or greater minimum inhibitory concentration values against the *Staphylococcus aureus* ATCC 25923 strain in the presence of human plasma [12–14]. This difference was proposed to be due to either protein binding or instability of the compounds. The application of liquid chromatography (LC) coupled with mass spectrometry (MS) as a powerful tool

for analysis of this class of compounds in biological fluids has been demonstrated in many studies [17–20]. Therefore, the purpose of this study was to assess the stability of these oxazolidinone derivatives in simulated gastric and intestinal fluids as well as in human plasma using a stability-indicating high-performance liquid chromatography (HPLC) procedure, and also to identify the chemical structures of the degradation products, if any, by MS analysis.

Materials and Methods

Materials

A series of TOZ compounds were prepared in our laboratory according to the reported method [11–14]. Human plasma samples were kindly donated by Kuwait Blood Bank, Kuwait and were stored at –25°C. All solvents were of HPLC grade and the reagents were of analytical grade. Water was purified by a Milli-Q device (Millipore, Bedford, Mass., USA).

HPLC and Chromatographic Conditions

The stability studies were conducted using an isocratic HPLC (Waters Alliance 2695 LC) connected to an Auto-sampler (Waters Association, Milford, Mass., USA). The chromatographic analyses were performed using a reversed-phase C₈ LC column (Waters, 250 mm length × 40 mm internal diameter, 5 µm particle size) and a mobile phase of acetonitrile/water (50/50 v/v) at a flow rate of 1 ml/min. A 20-µl aliquot of sample was injected into the system. The analysis run time was about 10 min and the eluent was monitored at λ_{254nm} using a Waters 996 PDA detector at ambient temperature. The peak areas of the eluted compounds were automatically measured by the Millennium software and were used to calculate the percent remaining and percent degradation of TOZ compounds.

Fig. 2. HPLC chromatograms of compound 1a in 0.1 M HCl at 37°C at 0 time (a), 3 h (b) and 24 h (c).

Fig. 3. HPLC chromatograms of compound 1b in 0.1 M HCl at 37°C at 0 time (a), 3 h (b) and 24 h (c).

Table 1. Stability of TOZ compounds in 0.1 M HCl solution at $37 \pm 1^\circ\text{C}$

Compound	X	R	K_{deg}	$t_{1/2}$	t_{90}
Lzd	O	HNCOCH ₃	0.0110 day^{-1}	63 days	9.5 days
PH-027	O		$5.53 \times 10^{-4} \text{ day}^{-1}$	1,254 days	190 days
1a	(CH ₃) ₃ OCON		0.180 h^{-1}	3.85 h	0.58 h
1b	(CH ₃) ₃ OCON		0.184 h^{-1}	3.76 h	0.57 h
1c	HCON		0.0198 h^{-1}	35 h	5.3 h
1d	HCON		0.0212 h^{-1}	32.7 h	4.95 h
1e	CCl ₃ CON		$5.53 \times 10^{-4} \text{ day}^{-1}$	1,254 days	190 days
1f	CCl ₃ CON		0.0166 day^{-1}	41.8 days	6.33 days
1g	PhCON		0.127 day^{-1}	5.46 days	20 h
1h	PhCO		0.0390 day^{-1}	17.8 days	2.7 days
1i			0.0152 h^{-1}	46 h	6.9 h
1j			0.110 day^{-1}	6.3 days	23 h

Lzd = Linezolid; K_{deg} = degradation rate constant; $t_{1/2}$ = degradation half-life; t_{90} = shelf life.

MS Analysis

MS analysis of compounds 1a and 1b and their respective degraded compounds was performed using tandem MS (Quattro LC, Micromass, Manchester, UK). The instrument tuning parameters were adjusted to 3.5 kV and 25 V for capillary voltage and cone voltage, respectively. The source and desolvation temperatures were 100 and 250°C , respectively. The MS scan was in the range of mass-to-charge (m/z) 100–500. Solutions of the examined compounds were prepared in the mobile phase (acetonitrile/water/formic acid, 80/20/0.025%, v/v/v) and injected directly into the mobile phase flowing to the ionization probe of the mass spectrometer at a flow rate of 50 $\mu\text{l}/\text{min}$ using positive electrospray ionization mode.

Procedures

Accelerated Stability Study of TOZ Compounds in Simulated Gastric and Intestinal Fluids. Stock solutions (1 mg/ml) of the TOZ compounds were separately prepared in acetonitrile. Eight

samples of 20- μl aliquots of each compound were diluted to 1 ml with either 0.1 M HCl (simulated gastric fluid) or phosphate buffer, pH 7.4 (simulated intestinal fluid). The solutions were well mixed, incubated at $37 \pm 1^\circ\text{C}$ and an aliquot of 20 μl of each solution was injected into the HPLC system. The samples were withdrawn at 0 time and then at 1-hour intervals for 6 h and then at 24 h in duplicates.

Stability Study of TOZ Compounds in Human Plasma. A 20- μl aliquot of each compound stock solution (1 mg/ml) in methanol/ H_2O (75/25) was diluted to 1 ml with plasma/water (1/1). Eight samples of each compound were prepared in diluted plasma and incubated at $37 \pm 1^\circ\text{C}$. A 50- μl aliquot of the plasma sample was withdrawn, at 0 time and then at 1-hour intervals for 6 h and at 24 h, and transferred into an Eppendorff tube and mixed with 250 μl of acetonitrile as protein precipitant. The samples were centrifuged at 980 g for 10 min and a 20- μl aliquot of the clear supernatant of each sample was injected into the HPLC system.

Table 2. Stability of TOZ compounds in phosphate buffer solution (pH 7.4) at $37 \pm 1^\circ\text{C}$

Compound	K_{deg} day ⁻¹	$t_{1/2}$, days	t_{90}
Lzd	0.072	9.625	1.46 days
PH-027	0.066	10.5	1.59 days
1a	0.05	13.9	2.1 days
1b	0.077	9	1.36 days
1c	0.0672	10.3	1.56 days
1d	0.072	9.625	1.46 days
1e	0.193	3.6	13 h
1f	0.028	24.8	3.75 days
1g	0.122	5.7	20.7 h
1h	0.166	4.18	15.2 h
1i	2.76×10^{-3}	251	38 days
1j	0.0387	17.9	2.71 days

Lzd = Linezolid; K_{deg} = degradation rate constant; $t_{1/2}$ = degradation half-life; t_{90} = shelf life.

Stability Profiles

The stability profiles were established by plotting the log% of remaining TOZ compound versus time (h). The degradation kinetic parameters, i.e. the rate of degradation (k_{deg}), degradation half-life ($t_{1/2}$), and shelf-life (t_{90}), were determined from the stability profiles using the formulas: $K_{\text{deg}} = 2.303 \times \text{slope (h}^{-1}\text{)}$, $t_{1/2} = 0.693/K_{\text{deg}}$ (h), $t_{90} = 0.105/K_{\text{deg}}$ (h), respectively.

Preparation of the Acid Degradation Products of Compounds 1a and 1b

A quantity (25 mg) of compounds 1a or 1b was dissolved in 1 ml acetonitrile and treated with 5 ml of 0.2 M HCl solution. The mixture was kept in a water bath thermostatically controlled at $37 \pm 1^\circ\text{C}$ for 3 h. Thin-layer chromatographic analysis showed that the compounds were completely hydrolyzed. The mixtures were then concentrated on a rotary evaporator and crystallized to give white solid masses. The collected powders were analyzed by MS measurements using a $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{formic acid}$ (80/20/0.025, v/v/v) solvent mixture at a concentration of 100 $\mu\text{g}/\text{ml}$. The degradation product of compound 1a [(R)-4-[2-fluoro-4-(2-oxo-5-[1,2,3]triazol-1-yl methyl-oxazolidin-3-yl) phenyl]-piperazine-1-ium hydrochloride] was analyzed by proton nuclear magnetic resonance ($^1\text{H-NMR}$). $^1\text{H-NMR}$ (DMSO-d_6 , 600 MHz): δ 8.84–8.94 (br, 2H, piperazinium $^+\text{NH}_2$), 8.17 (s, 1 H, triazole H), 7.76 (s, 1H, triazole H), 7.43 (dd, 1H, $J = 2.3$ Hz, 15.0 Hz, phenyl H), 7.16 (dd, 1H, $J = 2.3$ Hz, 9.6 Hz, phenyl H), 7.11 (t, 1H, $J = 9.1$ Hz, phenyl H), 5.11–5.17 (m, 1H, oxazolidinone H), 4.83 (d, 2H, $J = 4.99$ Hz, triazole- CH_2), 4.21 (t, 1H, $J = 8.7$ Hz, oxazolidinone H), 3.86 (dd, 1H, $J = 5.9$ Hz, 9.1 Hz, oxazolidinone H), 3.25 (br. s, 4H, piperazine $[\text{CH}_2]_2$), 3.17 (br. s, 4H, piperazine $[\text{CH}_2]_2$), MS (m/z) 347.5.

The degradation product of compound 1b [(R)-3-(3-fluoro-4-(piperazinium-1-yl) phenyl-5-(4-methyl-1H-1,2,3-triazol-1-yl) methyloxazolidin-2-one hydrochloride] was similarly analyzed by $^1\text{H-NMR}$. $^1\text{H-NMR}$ (DMSO-d_6 , 600 MHz): δ 8.80–8.94 (br,

Table 3. Stability of TOZ compounds in human plasma at $37 \pm 1^\circ\text{C}$

Compound	K_{deg}	$t_{1/2}$	t_{90}
Lzd	0.0166 day ⁻¹	41.7 days	6.3 days
PH-027	0.216 day ⁻¹	3.2 days	11.7 h
1a	0.0718 day ⁻¹	9.6 days	35 h
1b	0.0110 h ⁻¹	63 h	9.6 h
1c	0.0220 day ⁻¹	31.5 days	4.77 days
1d	0.144 day ⁻¹	4.8 days	17.5 h
1e	0.0110 day ⁻¹	62.7 days	9.5 days
1f	0.110 day ⁻¹	6.3 days	23 h
1g	0.166 day ⁻¹	4.2 days	15.2 h
1h	0.127 day ⁻¹	5.5 days	20 h
1i	0.0277 day ⁻¹	25 days	3.8 days
1j	5.53×10^{-3} day ⁻¹	125.4 days	19 days

Lzd = Linezolid; K_{deg} = degradation rate constant; $t_{1/2}$ = degradation half-life; t_{90} = shelf life.

2H, piperazinium $^+\text{NH}_2$), 7.86 (s, 1H, triazole H), 7.44 (dd, 1H, $J = 2.3$ Hz, 14.6 Hz, phenyl H), 7.16 (dd, 1H, $J = 2.4$ Hz, 9.7 Hz, phenyl H), 7.11 (t, 1H, $J = 9.2$ Hz, phenyl H), 5.06–5.11 (m, 1H, oxazolidinone H), 4.73 (d, 2H, $J = 6.1$ Hz, triazole- CH_2), 4.19 (t, 2H, $J = 9.2$ Hz, oxazolidinone H), 3.83 (dd, 1H, $J = 5.9$ Hz, 9.3 Hz), 3.25 (br. s, 4H, piperazine $[\text{CH}_2]_2$), 3.18 (br. s, 4H, piperazine $[\text{CH}_2]_2$), 2.22 (s, 3H, triazole- CH_3), MS (m/z) 361.5.

Results

The HPLC chromatograms of solutions of TOZ compounds in the mobile phase exhibited well-resolved peaks at retention times between 4.0 and 6.0 min (fig. 2, 3). With the exception of compounds 1a and 1b, TOZ compounds showed good stability in simulated gastric medium, without any degradation at simulated 37°C (table 1). On the other hand, compounds 1a and 1b underwent extensive degradation by acid hydrolysis. As shown in figure 2, the HPLC chromatogram of the acid-treated solution of compound 1a exhibited peaks of the TOZ compound and its acid-catalyzed degradation product at retention times 5.2 and 2.7 min, respectively. The intensity of the degradation product peak progressively increased with time. However, at the 24-hour interval, compound 1a was completely degraded and only a major peak for the degradation product appeared in the chromatogram. A similar behavior was noted in case of compound 1b, where complete degradation was observed after 24 h incubation at 37°C (fig. 3). The chromatograph-

Fig. 4. Acid-catalyzed hydrolysis of compounds 1a and 1b.

Fig. 5. MS scans of compound 1a (**a**) and degradation product of compound 1a (**b**).

ic peaks of compound 1b and the degradation product appeared at 5.5 and 2.7 min, respectively. Only a single peak for the degradation product was also observed in the chromatogram. The degradation pathways involving the acid cleavage of the t-butoxycarbonyl group of the examined compounds were suggested as shown in figure 4. A plot of the log% compound remaining versus time indicated first-order kinetics. Upon application of the kinetic analysis, the degradation kinetic parameters k_{deg} , $t_{1/2}$ and t_{90} were found to be 0.180 h^{-1} , 3.85 h, 0.58 h, respectively, for compound 1a, and 0.184 h^{-1} , 3.76 h and 0.57 h, respectively, for compound 1b (table 1). The degradation products were further identified by MS analysis, with complete disappearance of the molecular masses of compounds 1a and 1b at m/z 447.7 and 461.5, and the appearance of new molecular masses for the degradation products in MS at m/z 347.5 and 361.5. Representative mass spectra for compound 1a and its degradation product are shown in figure 5. The proposed chemical structures of the degraded products were in good agreement with MS data. On the other hand, the examined TOZ compounds were stable in phosphate buffer solutions and human plasma with remarkably low K_{deg} and high $t_{1/2}$ and t_{90} values (tables 2, 3).

Discussion

The developed HPLC method proved to be a stability-indicating procedure as it permits measurement of the examined TOZ compounds in the presence of their degradation products. However, it did not reveal the chemical structure of the degradation products, which could be identified by MS and confirmed by NMR analysis. The investigated oxazolidinones were remarkably stable in simulated biological fluids and human plasma at the simulated biological temperature of 37°C , with the exception of compounds 1a and 1b. These 2 TOZ compounds were completely degraded by acid hydrolysis in simulated gastric fluid within 24 h at 37°C . This may be due to the presence of the carbamate functionality, which is relatively more sensitive to acid-catalyzed degradation [21], compared to the amide functionality present in the other piperazinyl derivatives studied. The pathways for the acid-catalyzed degradation of compounds 1a and 1b were suggested. In case of compound 1a, the degradation product was identified as (*R*)-4-[2-fluoro-4-(2-oxo-5-[1,2,3]triazol-1-yl methyl-oxazolidin-3-yl) phenyl]-piperazine-1-ium hydrochloride. The MS analysis indicated that the degradation product of compound 1b is (*R*)-3-

(3-fluoro-4-(piperazinium-1-yl) phenyl-5-(4-methyl-1H-1,2,3-triazol-1-yl) methyloxazolidin-2-one hydrochloride. The findings of this study suggest that the previously observed 4-fold increase or greater MIC values for TOZ compounds, including 1a and 1b in human plasma (pH about 7.4) [12–14] may therefore be attributed to protein-binding pharmacokinetic factors rather than to poor stability of the compounds in human plasma.

Conclusion

The investigated oxazolidinones were stable in simulated biological fluids and human plasma at $37 \pm 1^{\circ}\text{C}$, with the exception of the carbamate-containing oxazolidinones, 1a and 1b. These TOZs were completely hydrolyzed in simulated gastric fluid with subsequent loss of the antibacterial activity. The lower stability of compounds 1a and 1b in gastric medium should be considered in formulation studies of these compounds for oral administration.

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