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LC-MS/MS identification of in vitro metabolites of a new H^+/K^+ ATPase inhibitor, KR-60436 produced by rat and human liver microsomes

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Abstract

The metabolism of a new H^+/K^+ ATPase inhibitor, KR-60436 [1-(4-methoxy-2-methyl-phenyl)-4-[(2-hydroxyethyl)amino]-6-trifluoromethoxy-2,3-dihydropyrrolo[3,2-*c*]quinoline] has been studied by LC-electrospray mass spectrometry. In vitro incubation of KR-60436 with rat and human liver microsomes in the presence of NADPH produced seven metabolites (M1–M7). M3–M6 were identified as *O*-demethyl-KR-60436, *O*-demethyl-pyrrole-KR-60436, *N*-dehydroxyethyl-KR-60436 and pyrrole-KR-60436, respectively, based on LC/MS/MS analysis with authentic standards. M1, M2 and M7 were tentatively identified as monohydroxylated-KR-60436, monohydroxylated-pyrrole-KR-60436 and *N*-dehydroxyethyl-pyrrole-KR-60436, respectively. The four principal metabolic pathways are characterized in KR-60436 metabolism: oxidation of dihydropyrrole ring to pyrrole, *O*-demethylation of methoxy group, hydroxylation of quinoline moiety and *N*-dealkylation of hydroxyethylamino group.

Keywords: KR-60436; In vitro metabolism; LC-ESI/MS; Liver microsomes

1. Introduction

Gastric H^+/K^+ ATPase inhibitors are the most potent suppressors of gastric acid and have been classified into two types: one type acts irreversibly and the other acts reversibly. Many benzimidazole derivatives, including omeprazole, exhibit powerful and long-lasting inhibition of gastric acid secretion due to irreversible inactivation of gastric H^+/K^+ ATPase. However, they may have several disadvantages, such as bacterial overgrowth [1] and possible carcinogeneicity [2], in clinical prac-

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tice. Recent efforts have turned on the development of reversible gastric H^+/K^+ ATPase inhibitors and some have been tested in clinical trials [3].

KR-60436, 1-(4-methoxy-2-methylphenyl)-4-[(2-hydroxyethyl)amino]-6-trifluoro methoxy-2,3-dihydropyrrolo[3,2-*c*]quinoline, has been newly synthesized by the Metabolic Diseases Research Laboratory of Korea Research Institute of Chemical Technology (KRICT, Taejeon, South Korea) as a potent reversible gastric H^+/K^+ ATPase inhibitor. KR-60436 is currently being investigated in a pre-clinical study for development as an anti-ulcer agent [4–6]. In previous pharmacokinetic studies of KR-60436 [7,8], the biliary and urinary excretion of KR-60436 were 3% and $<$ 0.03%, respectively, after a single oral dose of KR-60436 to male rats, indicating that KR-60436 might undergo significant hepatic metabolism. The purpose of this study was to profile and identify the metabolites of KR-60436 produced by rat and human liver microsomes using LC/electrospray-mass spectrometry (ESI-MS).

2. Experimental

2.1. Chemicals

KR-60436 and four possible metabolites, 1-(4-methoxy-2-methylphenyl)-4-[(2-hydroxyethyl) amino]-6-trifluoromethoxypyrrolo[3,2-*c*]quinoline (pyrrole-KR-60436), 1-(4-hydroxy-2-methylphenyl)-4-[(2-hydroxyethyl)amino]-6-trifluoromethoxy-2,3-dihydropyrrolo [3,2-*c*] quinoline (*O*-demethyl-KR-60436), 1-(4-hydroxy-2-methylphenyl)-4-[(2-hydroxyethyl) amino]-6-trifluoromethoxypyrrolo[3,2-*c*]quinoline (*O*-demethyl-pyrrole-KR-60436) and 1-(4-methoxy-2-methylphenyl)-4-amino-6-trifluoromethoxy-2,3-dihydropyrrolo[3,2-*c*]quinoline (*N*-dehydroxyethyl-KR-60436) were synthesized at the Metabolic Diseases Research laboratory of the KRICT. $NADP^+$, glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase and acetic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Pooled male rat and human liver microsomes were obtained from Gentest (Woburn, MA). Methanol and acetonitrile (spectroscopy grade) were obtained from Burdick and Jackson Inc. (Muskegon, MI) and the other chemicals were of the highest quality available.

2.2. KR-60436 metabolism in rat and human liver microsomes

The metabolic experiments were performed at 37 °C for 30 min with a reaction mixture consisting of rat or human liver microsomal protein (0.25 mg), 1 mM $NADP^+$, 10 mM G-6-P, 1 U G-6-P dehydrogenase, 5 mM magnesium chloride, potassium phosphate buffer (pH 7.4; 50 mM) and 5 μM substrate in a final volume of 0.5 ml. The incubation was stopped by the addition of 50 mM NaOH (50 μl) and methylene chloride (1 ml). After mixing and centrifugation, the resulting organic layer was evaporated under nitrogen and the residue was dissolved in 0.1 ml of 30% acetonitrile. The aliquot (10 μl) were analyzed by LC-MS to identify unchanged KR-60436 and its metabolites. The blanks were conducted in the absence of NADPH-generating system. The recoveries of KR-60436, *O*-demethyl-KR-60436, *O*-demethyl-pyrrole-KR-60436, pyrrole-KR-60436 and *N*-dehydroxyethyl-KR-60436 were 96.6 ± 3.1, 94.2 ± 3.4, 93.2 ± 3.9, 93.9 ± 4.5 and 95.0 ± 4.0%, respectively

2.3. LC-ESI-MS/MS analysis

All experiments were performed with a Shiseido Nanospace SI-2 system and a Quattro-LC tandem quadrupole mass spectrometer (Micromass UK Ltd., Manchester, UK) equipped with an electrospray ion source. For the identification of metabolites, a Discovery RP-Amide column (5 μm, 2.1 mm i.d. × 15 cm, Sigma Aldrich) eluting with acetonitrile and water both containing 0.1% acetic acid at a flow rate of 0.2 ml/min and a gradient of 15–40% acetonitrile over 20 min followed by isocratic elution at 40% acetonitrile for an additional 10 min. The source and desolvation temperature were held at 120 and 230 °C, respectively. The capillary and cone potential were 3.0 kV and 40 V, respectively. The molecular ions of the analytes were extracted and fragmented by

collision-induced dissociation, which was achieved with argon collision gas and collision energy of 48 eV. Product ions were scanned in the m/z range 100–600, and the spectra were collected in the form of continuum data.

3. Results and discussion

From the incubation of KR-60436 with rat and human liver microsomes in the presence of NADPH-generating system, unchanged KR-60436 and seven metabolites were profiled, characterized and tentatively identified using LC-MS analysis (Fig. 1). Metabolites were named as M1–M7 in the order of elution. No metabolites were produced in the absence of NADPH, thus indicating the involvement of cytochrome P450 (CYP) enzymes. LC/ESI-MS/MS analysis of unchanged KR-60436 and its metabolites (M1–M7) produced the informative and prominent product ion spectra (Fig. 2). The product ion spectrum of KR-60436, having a protonated molecular ion $[M+H]^+$ at m/z 434, showed the prominent product ions at m/z 390 (the loss of hydroxyethyl group), 350 (the loss of trifluoromethoxy group) and 122 (2-methyl-4-methoxyphenyl ring).

M6, a major metabolite peak, showing $[M+H]^+$ at m/z 432, was identified as pyrrole-KR-60436 based on co-chromatography and MS/MS data of the authentic standard. MS/MS spectrum of M6 showed the prominent MS/MS fragments at m/z 388 (the loss of hydroxyethyl group), 348 (the loss of trifluoromethoxy group) and 122 (2-methyl-4-methoxyphenyl ring). The incubation of M6 with rat liver microsomes in the presence of NADPH produced three metabolites, i.e. M2, M4 and M7 (data not shown).

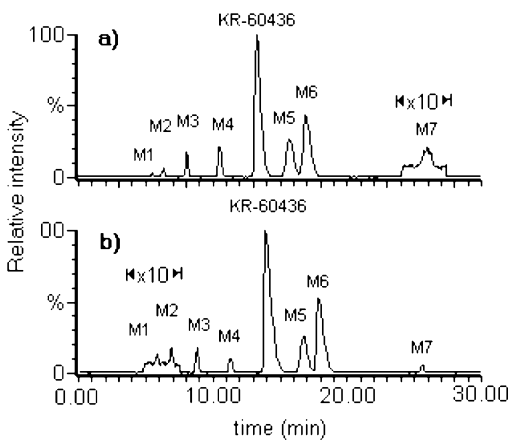

Fig. 1. LC-ESI-MS ion chromatograms of (a) the rat liver microsomal incubation and (b) the human liver microsomal incubation of KR-60436 in the presence of NADPH-generating system.

M3 and M5 having $[M+H]^+$ at m/z 420 (14 amu less than the parent KR-60436) and 390 were confirmed as *O*-demethyl-KR-60436 (44 amu less than parent KR-60436) and *N*-dehydroxyethyl-KR-60436, respectively, in comparison with co-chromatography and MS/MS spectra of the authentic standards. M3 (*O*-demethyl-KR-60436), resulting from the cleavage at methoxy group, produced MS/MS fragments at m/z 108 (2-methyl-4-hydroxyphenyl ring), 334 (the loss of trifluoromethoxy group) and 374 (a loss of hydroxyethyl group). M5 (*N*-dehydroxyethyl-KR-60436), resulting from *N*-dealkyation at 2-hydroxyethylamino group, showed MS/MS fragments at m/z 122 (2-methyl-4-methoxyphenyl ring) and m/z 306 (the loss of trifluoromethoxy group).

M4 showing $[M+H]^+$ at m/z 418 was identified as *O*-demethyl-pyrrole-KR-60436 by co-chromatography and MS/MS spectral data of the authentic compound. M4 was formed by both *O*-demethylation and dihydropyrrole-oxidation of KR-60436 because M4 was also identified from the incubation of pyrrole-KR-60436 or *O*-demethyl-KR-60436 with rat liver microsomes in the presence of NADPH.

M7, having a protonated molecular ion of m/z 388, produced MS/MS fragment ions at m/z 122 (2-methyl-4-methoxyphenyl ring) and m/z 304 (loss of trifluoromethoxy group). M7 was detected in the incubations of pyrrole-KR-60436 or *N*-dehydroxyethyl-KR-60436, as well as KR-60436 with liver microsomes. From these results, M7 was tentatively identified as *N*-dehydroxyethyl-pyrrole-KR-60436.

M1 possessed a molecular weight 16 more than the parent KR-60436 ($[M+H]^+$ at m/z 450), suggesting the introduction of an oxygen atom to KR-60436. The product ions at m/z 406 (the loss

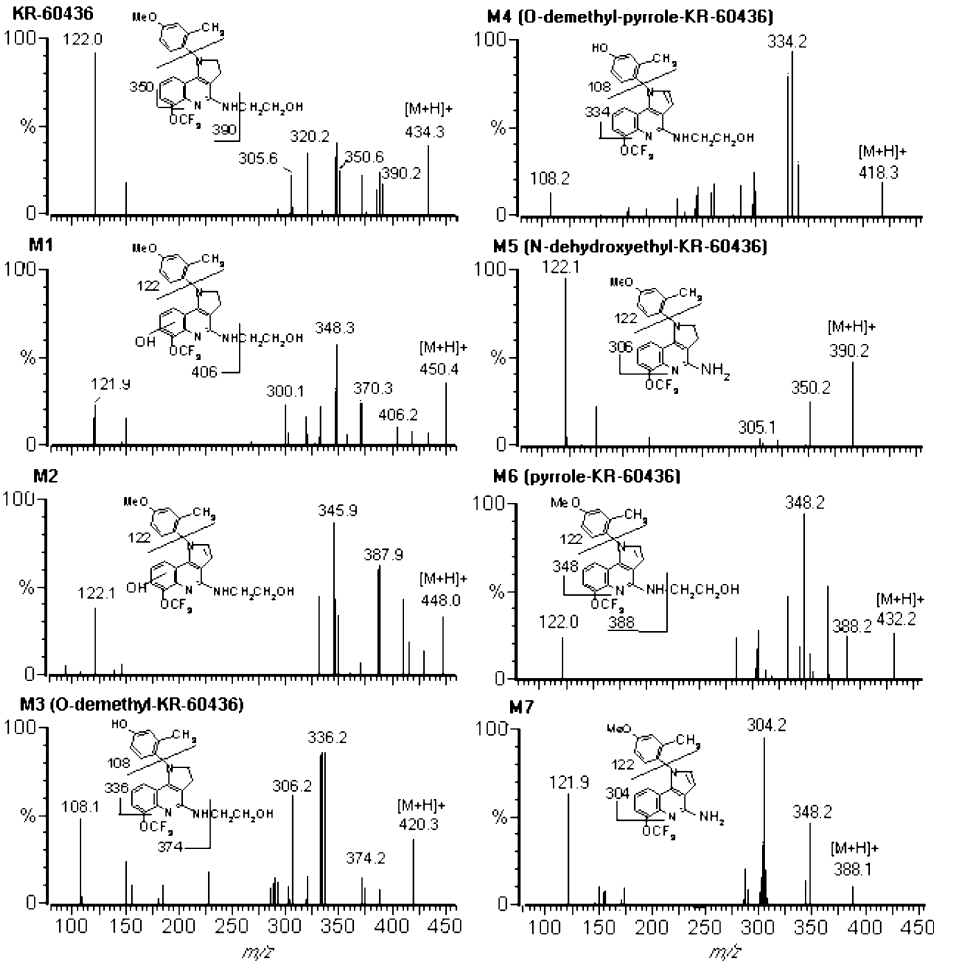

Fig. 2. ESI product ion mass spectra of KR-60436 and the metabolites, M1–M7.

of hydroxyethyl group), 348 (the loss of trifluoromethoxy group and water) and 122 (2-methyl-4-methoxyphenyl ring) suggested that M1 might be KR-60436 hydroxylated at the quinoline moiety (OH-KR-60436), though the regiochemistry of the functionality could not be determined.

Protonated molecular ion of M2 was observed at m/z 448, which was 16 amu greater than pyrrole-KR-60436 (M6), suggesting the introduction of an oxygen atom and dehydrogenation of two hydrogen atoms in KR-60436. The characteristic fragment ions at m/z 346 (the loss of trifluoromethoxy group and water) and 122 (2-methyl-4-methoxyphenyl ring) were clearly observed and the incubation of pyrrole-KR-60436 with liver microsomes formed M2. Therefore, M2 was tentatively identified as the hydroxylation product of pyrrole-KR-60436 (OH-pyrrole-KR-60436).

Based on these results, the major metabolic pathways of KR-60436 metabolism in rat and human liver microsomes are summarized in Fig. 3. KR-60436 was extensively metabolized by four principal metabolic pathways: (a) oxidation of dihydropyrrole moiety to pyrrole, (b) *O*-demethylation of methoxy group, (c) mono-hydroxylation of the quinoline moiety and (d) *N*-dealkylation of hydroxyethylamino side chain. The oxidation of dihydropyrrole to pyrrole forming M2, M4 and M7 as well as M6, was the major pathway of

KR-60436 metabolism. M1 and M2 were structurally assumed as the hydroxylated metabolites of quinoline moiety of KR-60436 and pyrrole-KR-60436, respectively. The hydroxylation of quinoline moiety has been described in the metabolism of a similar acylquinoline proton pump inhibitor, DBM-819 to 8-hydroxy-DBM-819 [9]. *O*-demethylation step produced an active metabolite, *O*-demethyl-KR-60436 having the same H^+/K^+ ATPase inhibitory activity as KR-60436 [8].

4. Conclusion

The new proton-pump inhibitor KR-60436 was metabolized in rat and human liver microsomes by means of four oxidative metabolic pathways. Seven metabolites (M1–M7) formed in both species were pyrrole-KR-60436, *O*-demethyl-KR-60436, *O*-demethyl-pyrrole-KR-60436, *N*-dehydroxyethyl-KR-60436, *N*-dehydroxyethyl-pyrrole-KR-60436, OH-KR-60436 and OH-pyrrole-KR-60436.

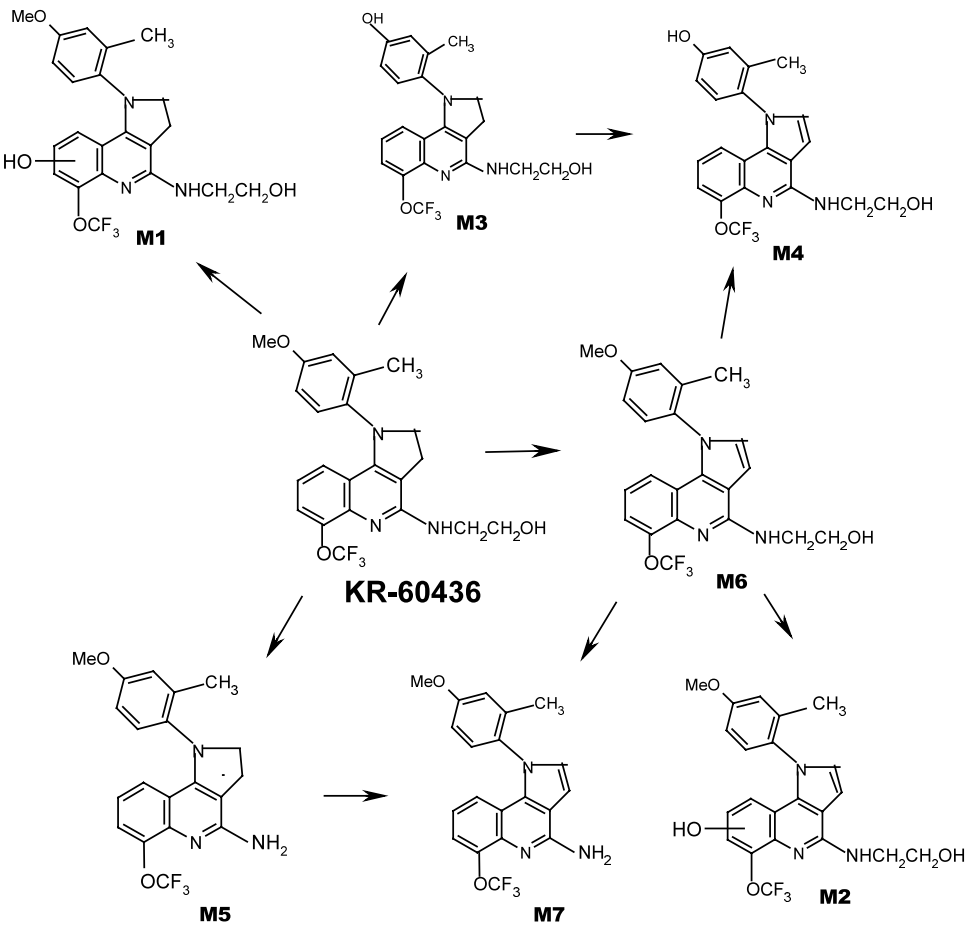

Fig. 3. Possible metabolic pathways of KR-60436 in rat and human liver microsomes.

Acknowledgements

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