Identification of IY81149 and Its Metabolites in the Rat Plasma Using the On-Line HPLC/ESI Mass Spectrometry

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Reversed-phase high-performance liquid chromatography/mass spectrometry (HPLC/MS) with an electrospray ionization (ESI) interface was applied to the identification of metabolites of IY 81149 in the rat plasma. Fragments obtained using collision-induced dissociation (CID) in both positive and negative modes were utilized to elucidate the structure of metabolites. The eluent from the conventional HPLC column was split and directly introduced into an ESI-mass spectrometer for the identification of the structures. The CID technique allowed the sensitive identification of sulfonyl-IY81149 and hydroxy-IY81149 from the rat plasma.

Key words : IY81149, Metabolites, H^+/K^+-ATPase inhibitor, HPLC, Electrospray ionization (ESI), Mass spectrometry (MS), Collision induced dissociation (CID)

INTRODUCTION

The most common analytical methods for the identification and separation of the metabolites with a wide polarity range are the chromatographic and/or mass spectrometric methods, singly and/or as hyphenated methods. Mass spectrometry is a powerful technique which has been widely used for the structural elucidation of drug metabolites. In general, mass spectrometric procedures need the separation of the various metabolites from biological fluids, and the characterization of individual components. The procedure is labor-intensive and requires large volumes of fluids, due to the low concentration of these compounds in plasma, urine, and bile samples. The direct combination of mass spectrometry with chromatographic techniques, which is called the "on-line" in this paper, offers effective alternative for metabolic studies which involve the analysis of metabolites in complex biological matrices, such as cell culture medium, plasma, bile and urine. For example, for a metabolic study of omeprazole, which is an H^+/K^+-ATPase inhibitor, HPLC is successfully interfaced with mass spectrometry using thermospray (TSP) ionization (Beattie *et al.*, 1989) and atmospheric pressure ionization (API) (Weidolf *et al.*, 1992) techniques.

IY81149, a substituted benzimidazole, is a new candidate drug that is an H^+/K^+-ATPase inhibitor designed for the treatment of gastric ulcers. Substituted benzimidazoles are superior to histamine H_2-receptor antagonists in terms of the shorter healing rates of gastric and duodenal ulcers (Black, 1993; Lindberg *et al.*, 1990; McArthur *et al.*, 1985; McTavish *et al.*, 1991). IY81149, which is under development by Il Yang Pharmacy Co. (Seoul, Korea), has been proved by a series of animal studies to be a potent and very safe antiulcer agent (Kim *et al.*, 1997). When a drug is administered to an animal or human, the administered drug is converted into more polar compounds which can then be rapidly excreted. Since IY81149 and its metabolites are labile both in organic solvents and in the aqueous phase, they are required to be analyzed immediately after the biological samples are collected. This paper describes an on-line HPLC/ESI mass spectrometric method for the identification of IY81149 and its metabolites from rat plasma. A conventional HPLC column was interfaced to the mass spectrometer, and the analytes eluted from the HPLC were analyzed by the collision-induced dissociation (CID)-ESI MS method. CID produces fragment ions when the difference ("CapEx" voltage) between the voltage applied to capillary exit and that applied to the first skimmer in the electrospray source is sufficient. This method has

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advantages such as ease of use, cost and reliability compared with traditional MS/MS experiments. The fragments resulting from the CapEx variance can be utilized to identification of the structures. The on-line coupling could be easily accomplished through the use of a post-column flow splitter with a split ratio of 1:150, so that 7 μl/min entered the electrospray system while the majority of the column effluent went to waste or collect. IY81149 and its metabolites were separated by reversed-phase HPLC and analyzed by the positive and negative ESI/mass spectrometry.

MATERIALS AND METHODS

Drug and chemicals

IY81149 was provided by Il Yang Pharmacy Co. (Seoul, Korea). Methylene chloride and methanol were obtained from Merck (Darmstadt, Germany), and ammonium acetate was purchased from Kanto Chemical Co. (Tokyo, Japan). All other chemicals and solvents used were of the highest purity grade available and were used without further purification. Male Sprague-Dawley rats (200~250 g) were obtained from the Korea Food and Drug Administration, Seoul, Korea.

Drug administration and sample preparation

IY81149 was dissolved in a solution of 0.9% sodium chloride for intravenous administration to rats. IY81149 (100 mg/kg) was intravenously injected to male Sprague-Dawley rats, weighing 200~250 g at the time of the study. After 1 hour from administration of IY81149, blood was collected in a tube treated with heparin. Plasma obtained by centrifugation was kept frozen. IY81149 and its metabolites were extracted from 500 μl of the plasma with 1 ml of methylene chloride after adding 100 μl of 0.05 M ammonium acetate buffer (pH 7.3). The organic phase containing the analytes was evaporated using N_2 gas and the residue was dissolved in 50 μl of the mobile phase before injection into the HPLC system.

High-performance liquid chromatography/Mass spectrometry

The mass spectrometry system was based on a HP 5989A MS Engine (Hewlett-Packard, Palo Alto, CA, USA) mass spectrometer with a HP Model 59987A Electrospray MS system. For the on-line system, a Yong-In Model 910 solvent delivery system (YongIn Scientific Co., Seoul, Korea) was used with a Rheodyne 7725 injector (20 μl sample loop). A 3.9 mm × 300 mm, 10 μm μ-Bondapak C_{18} analytical column (Waters Chromatography, Milford, MA, USA) was used, and the elution was accomplished with a mixture of methanol and 0.05 M ammonium acetate (pH 7.3) (55:45 v/v) at a flow rate of 1.0 ml/min. The effluent was connected to a post-column splitter (LC Packings Inc., Switzerland) with a 50 mm length of a 0.05 mm i.d. PEEK tubing (LC Packings Inc., Switzerland). The post column splitter provided a split ratio of 1:150 to deliver a rate of 7 μl/min into the electrospray needle. Samples from the post-column splitter were introduced into the ESI interface through a heated nebulizer probe (150°C for the positive mode and 100°C for the negative mode) using nitrogen gas for the positive mode and air for the negative mode as a nebulizing gas. The CapEx voltage was varied over the range of 100~300 V.

RESULTS AND DISCUSSION

IY81149 and its metabolites were separated by isocratic reversed-phase HPLC. The total ion chromatograms (TIC) obtained by on-line HPLC/positive ESI mass spectrometry were shown in Fig. 1(a). And the

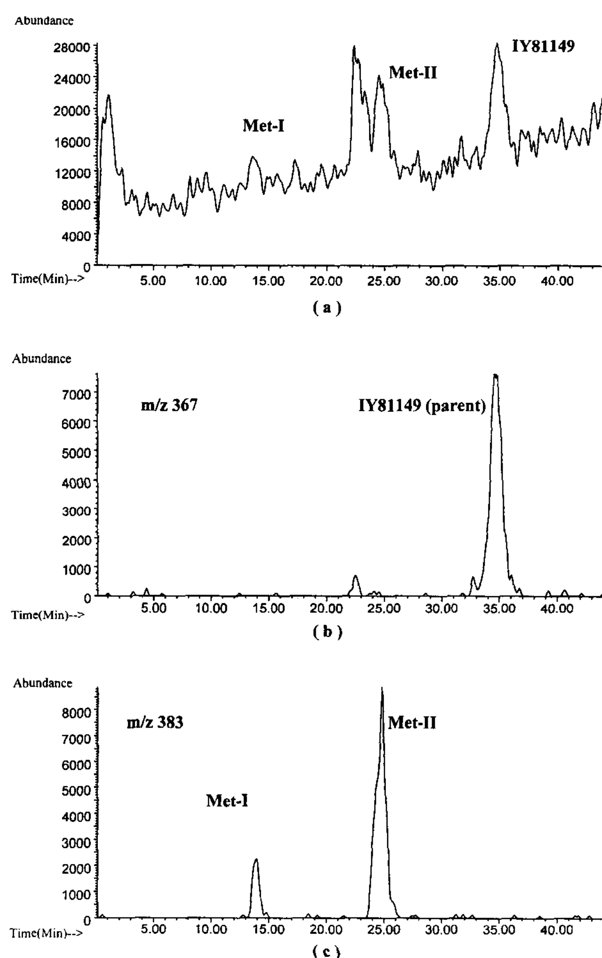

Fig. 1. HPLC/ESI-MS analysis of the plasma from 5~60 min timed collection period. (a) Total ion chromatogram for m/z 100~500; (b) selected ion chromatogram trace for m/z 367; (c) selected ion chromatogram for m/z 383

selected ion chromatogram for the parent drug ($[M+H]^+$, m/z 367)) and the supposed major metabolites ($[M+H]^+$, m/z 383) were shown in Fig. 1(b) and 1(c). Two of the major metabolites at retention times of 13.7 min and 24.8 min were designated Metabolite I (Met-I) and Metabolite II (Met-II), and were supposed to be the hydroxy-IY81149 and sulfonyl-IY81149, respectively. The structures of IY81149 and proposed metabolites were shown in Fig. 2. The identities of Met-I and -II can be proposed by the elution order and UV spectra obtained from the HPLC system, and the mass spectra obtained from the CID experiments. Because of the low numbers of fragments, the structural information provided by the positive ionization ESI, using typical CID voltages, was somewhat limited. Electrospray can provide valuable structural information about pure samples, or chromatographically separated components of mixtures, through in-source CID. In the instrument used here, CID occurs in the space between the capillary exit and the first skimmer in the API-electrospray source, and produces fragment ions when the CapEx voltage is sufficient. This differential potential generates enough energy to break bonds by accelerating ions into collisions with molecules of drying gas (Hewlett-Packard, 1994). After the fractions of the parent and metabolites were collected separately, the structural identification by CID with varying the CapEx voltage was performed. The changes in the fragmentation patterns in the mass spectra (in the positive ionization mode) of IY81149, Met I, and Met II, by the variation of the CapEx voltage, were shown in Fig. 3. The structural information about the metabolites thus obtained in the positive ion mode was of higher abundance than in the negative ion mode. The spectra obtained for the parent compound

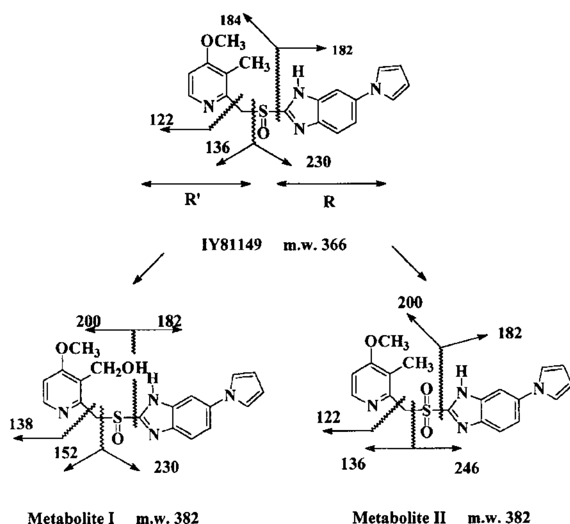

Fig. 2. Proposed metabolites of IY81149

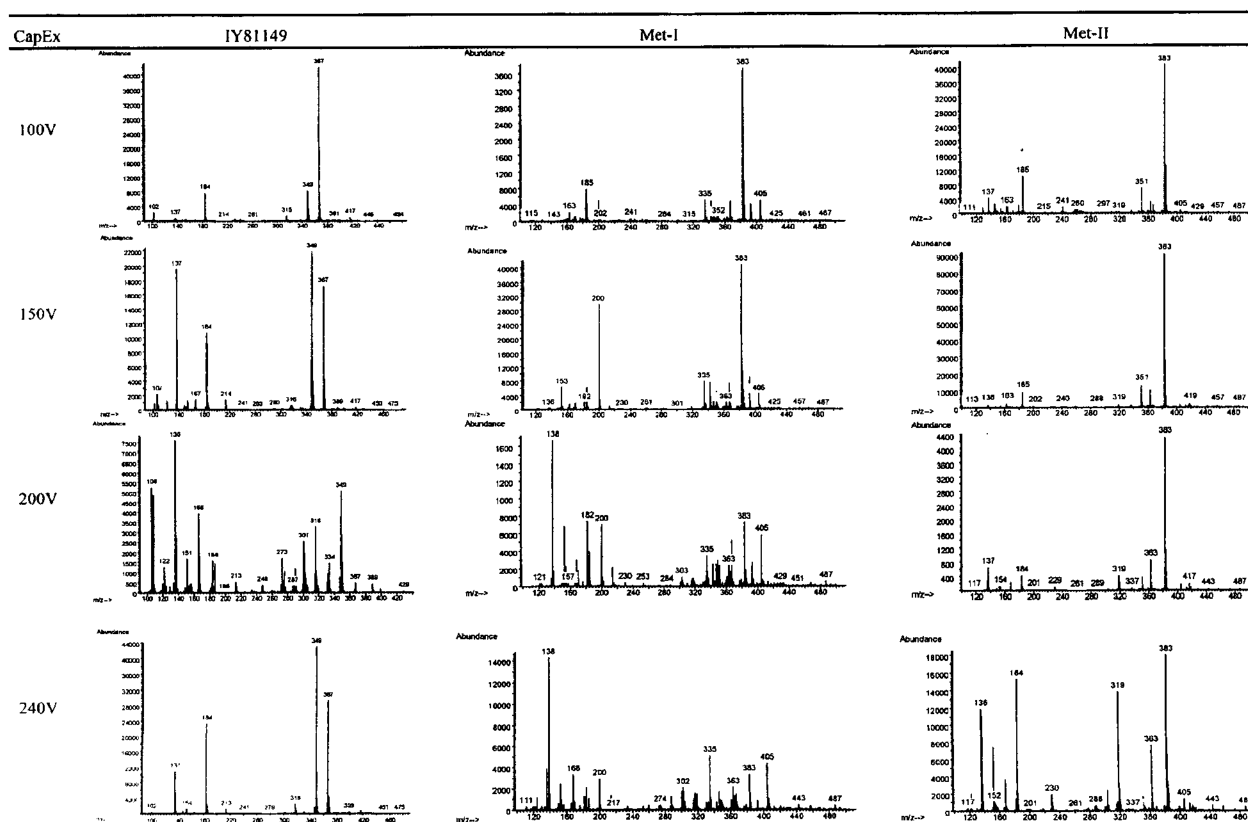

Fig. 3. Mass spectrum change of IY81149 and its metabolites by the variation of the CapEx voltage

(IY81149), Met-I (hydroxy-IY81149) and Met-II (sulfonyl-IY81149) were markedly different using a CapEx voltage of 200 V and 240 V in the positive mode. Unchanged IY81149 (the parent drug), Met-I, and Met-II produced simple 'one-peak' spectra at low CapEx voltages with $[M+H]^+$ ions at m/z 367, 383 and 383 without any further significant fragmentation, respectively. In the high voltage spectra, the ion corresponding to the pyridyl group half of the molecule was distinctly fragmented as shown in Fig. 3. The cleavage of IY81149 to form the pyridyl (substructure R' in Fig. 2) plus its sulfinyl (-S=O) group accounts for the ion at m/z 184. The loss of water from m/z 184 resulted in the ion at m/z 166. The losses of -SO and of $-CH_2SO$ were detected at m/z 137, which was the loss of sulfinyl (-S=O) from m/z 184 plus 1 mass unit, and m/z 122, respectively. In this study, the pyridyl (substructure R' in Fig. 2) groups were detected at an increment of 1 mass unit, this situation was common to the parent, Met-I and Met-II.

In the negative mode (Fig. 4), there was no useful fragmentation to elucidate the structures of these molecules, even though a high CapEx voltage was applied. The molecular anion at m/z 366, the sulfinyl-benzimidazol (substructure R) anion at m/z 230, and the benzimidazole portion at m/z 182, were observed.

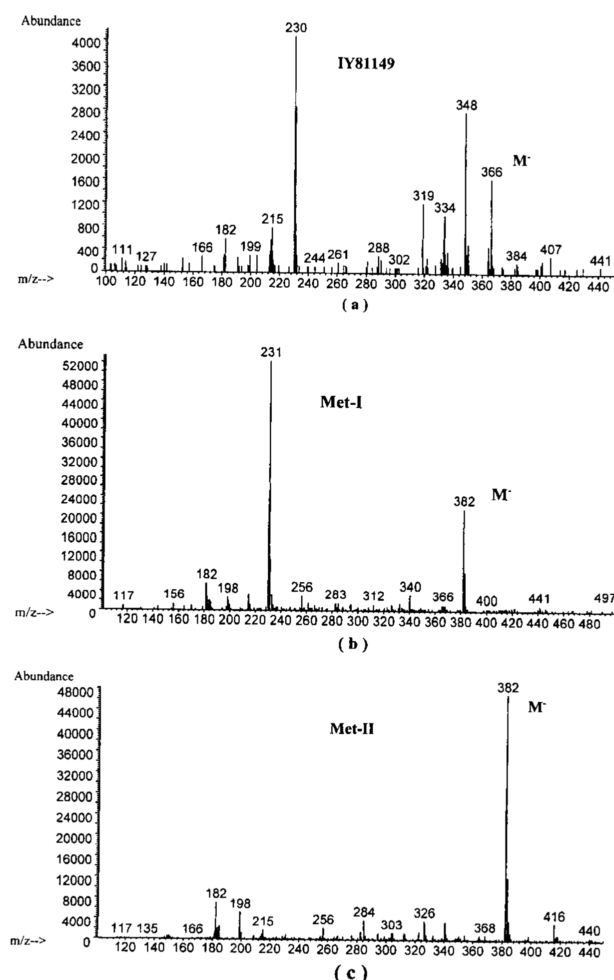

Fig. 4. Negative ESI mass spectra of IY81149 and its metabolites (CapEx voltage=240 V)

Metabolite I

The structure of the proposed Met-I was shown in Fig. 2. The spectrum for the putative Met-I produced an MH^+ ion at m/z 383, spectrum 16 Da higher than that of the parent drug, as a base peak in the CapEx=100 spectrum (Fig. 3(b)). From the spectra obtained using a high CapEx voltage (150 V to 240 V) (Fig. 3(a)), structural information was elucidated. From the difference of 16 mass unit, addition of oxygen to the parent molecule (IY81149) was proposed. A major fragment at m/z 200, which was 16 mass unit higher than the ion at m/z 184 (substructure R' plus sulfinyl) from the parent compound, correspond to loss of benzimidazole (substructure R). The ion corresponding to the pyridyl group in the molecule was moved up 16 mass unit to m/z 184 as a result of the hydroxy being situated on the pyridyl methyl or sulfonyl (O=S=O). The site of the hydroxyl (-OH) group could be assigned to the pyridyl methyl due to the presence of the ions at m/z 153, which was m/z 152 plus 1 mass unit, and 138, which corresponded respectively to the ions at m/z 137, and 122 which are detected in the spectrum of the parent drug (Fig. 3). This assumption was confirmed by the presence of the ions at m/z 230 and 182 in a negative ion spectrum obtained using a high CapEx potential (Fig. 4(b)), and confirmed that the benzimidazole portion of the molecule was not changed.

Metabolite II

Met-II was assigned as the sulfonyl (O=S=O)-IY81149 as shown Fig. 3(c). In the typical low-voltage (CapEx=100 V) positive-ion spectrum, the base peak was detected at m/z 383 which represented the $[M+H]^+$ ion. The spectrum obtained at high CapEx voltages (CapEx=200 V and 240 V) gave abundant fragmentation for the structural interpretation. The ion at m/z 184 corresponded to lose of oxygen at the position of the sulfonyl from the R' portion plus the sulfonyl (m/z 200). An ion is observed at m/z 136 or 137, which corresponded to the methylene-pyridyl break like as IY81149. This proved an assumption that the site of oxygen in the Met-I was situated on the pyridyl group, and the Met-II contained the sulfonyl group. In the negative ion mode, the ion fragment observed at m/z 182 results from the intact benzimidazole portion and the ion at m/z 382 is the $[M]^-$ anion. Also from the chromatographic data, this assumption was proved from that the sulfonyl metabolite in the omeprazole

metabolism study by the HPLC/MS (Beattie *et al.*, 1989; Weidolf *et al.*, 1992) and HPLC (Kobayashi *et al.*, 1992; Andersson *et al.*, 1993) being eluted closely before a parent drug.

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