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Short communication

Simple method for determination of the active metabolite of the inotropic drug pimobendan in rat liver microsomes

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Abstract

A rapid and sensitive high-performance liquid chromatographic method for quantitation of the *O*-demethylated active metabolite formed in a liver microsomal assay system has been developed. The metabolite was separated on an Inertsil ODS-2 column and quantitated by fluorescence detection (excitation at 338 nm, emission at 405 nm). The retention times for pimobendan and its metabolite were 5.6 and 2.8 min, respectively. The intra- and inter-assay relative standard deviations in the measurement of pimobendan *O*-demethylase activity at the substrate concentrations of 1 μ*M* and 500 μ*M* were 2.0%, 6.8%, 2.1% and 5.6%, respectively, and the limit of detection was 0.1 ng for the demethylated metabolite.

Keywords: Pimobendan

1. Introduction

Pimobendan, 4,5-dihydro-6-[2-(4-methoxyphenyl)-5-benzimidazolyl]-5-methyl-3(2H)-pyridazinone, is a member of a new class of inotropic drugs; it augments Ca^{2+} sensitivity [1,2] and inhibits phosphodiesterase-III activity [3] in cardiomyocytes, and is used for the treatment of patients with heart failure [4–6]. The drug is extensively metabolized by oxidation in the liver (Fig. 1) [7]. The oxidatively demethylated metabolite also augments Ca^{2+} sensitivity and inhibits phosphodiesterase-III activity, although the mechanism of Ca^{2+} sensitization is different from that of the parent compound, pimobendan [2]. The *O*-demethylated metabolite[1] seems to contribute substantially to the pharmacological effects of pimobendan [8]. However, there is no information concerning the enzyme involved in the oxidative metabolism of pimobendan at present. It seems clinically important to characterize the enzyme(s) responsible for the metabolism of pimobendan, and as a first step towards this goal, we

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[1]*O*-Demethylated pimobendan; 4,5-dihydro-6-[2-(4-hydroxyphenyl)-5-benzimidazolyl]-5-methyl-3(2H)-pyridazinone, UD-CG212.

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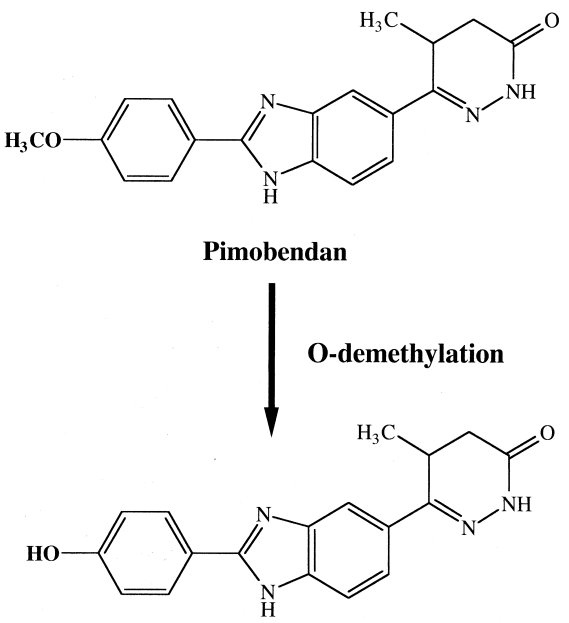

Fig. 1. Metabolic pathway and structure of pimobendan.

have developed a simple and sensitive method for determination of the pimobendan metabolite.

2. Materials and methods

2.1. Drugs and chemicals

Pimobendan and its metabolite were provided by Boehringer Ingelheim Pharma, Germany. Analysis by high-performance liquid chromatography (HPLC) indicated that the purity of both compounds was more than 99.5%. NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast (Tokyo, Japan). HPLC-grade methanol and acetonitrile were from Wako (Osaka, Japan). All other chemicals were of the highest grade commercially available.

2.2. Preparation of microsomes

Male Sprague–Dawley rats (8 weeks old) were housed in wire-bottomed stainless steel cages in a temperature- and light-controlled room (25°C, 12 h light/dark cycle). The rats were maintained on commercial rat chow (CE-2; Nippon Clea, Tokyo, Japan) and tap water for 7 days prior to sacrifice. Microsomes were prepared according to the methods previously described [9]. After the determination of protein concentration according to Lowry et al. [10], the microsomes were suspended in 50 m*M* potassium phosphate (pH 7.4) containing 0.1 m*M* EDTA (pH 7.4) and kept at −80°C until use.

2.3. Incubation and sample preparation

A typical incubation mixture contained 0.1 m*M* potassium phosphate (pH 7.4), 0.1 m*M* EDTA (pH 7.4), an NADPH-generating system (0.33 m*M* NADP, 8 m*M* glucose 6-phosphate, 0.1 unit of glucose 6-phosphate dehydrogenase, 6 m*M* $MgCl_2$), substrate and a suitable amount of microsomes in a final volume of 1 ml. The concentrations of substrate used are specified in the figure legends. After the pimobendan solution in methanol was transfered into test tube, the methanol was evaporated before the addition of other components. The reaction was initiated by the addition of the NADPH-generating system. The mixture was incubated at 37°C with shaking for an appropriate period, then the reaction was stopped by the addition of 1 ml of a mixture of 0.2 *M* potassium phosphate (pH 2.0)–2 *M* HCl (1:1, v/v). The mixture was centrifuged at 1400 *g* for 10 min and the supernatant was transferred into a 1 ml syringe, filtered through a Chromatodisk, and subjected to an HPLC as described below. The injection volume of the sample was 50 μl.

2.4. Calibration curve

The solution of *O*-demethylated pimobendan in methanol was used as a standard solution. To clarify the range of linearity, twelve different concentrations of standard solution (2 ng/ml to 4 μg/ml) were prepared by the dilution of *O*-demethylated pimobendan solution (200 μg/ml) with methanol, and the solutions were subjected directly to a HPLC system.

For the estimation of the metabolite formed, after 10, 50 and 100 μl of standard solution in methanol (50 μg/ml) were added to the reaction mixture, the sample was processed as described in Section 2.3. The amount of the metabolite formed was quantitated using the peak area. The activity of pimoben-

dan *O*-demethylase was calculated using a molecular mass of 320.35.

2.5. Chromatographic conditions

The HPLC system consisted of two flow pumps (Models 655 and 655A-11, Hitachi, Tokyo, Japan), a fluorescence detector (Model RF-530, Shimadzu, Tokyo, Japan), a chromato integrator (Model D-2000, Hitachi), a system controller and an Inertsil ODS-2 column (ϕ=5 μM, 15 cm×4.6 mm, GL Science, Tokyo, Japan). The mobile phase, methanol–acetonitrile–ammonium acetate (0.6%) (3:1:4, v/v/v), was delivered at a flow-rate of 1.3 ml/min. HPLC analysis was performed at 40°C. The excitation and emission wavelengths of the detector were set at 338 nm and 405 nm, respectively. The time constant of the fluorescence detector was 1.5 s. To increase the fluorescence intensity of the metabolite, a mixture of methanol–42% phosphoric acid (3:2, v/v) was added at a flow-rate of 0.3 ml/min to the column effluent through a T-connector, located upstream from the detector.

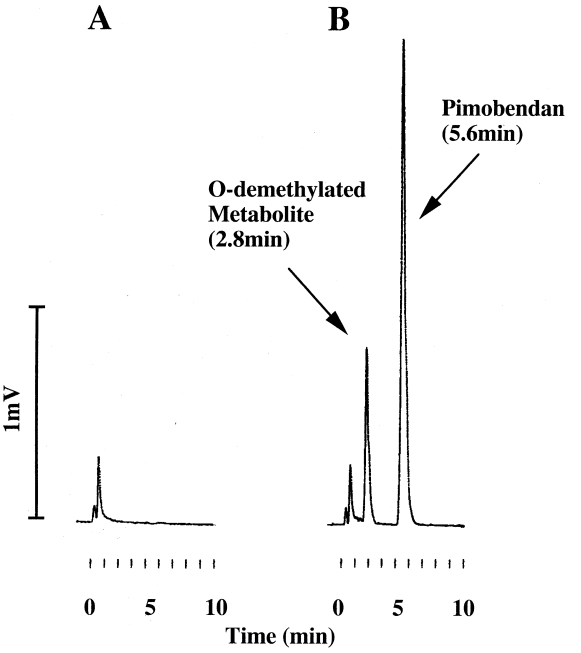

Fig. 2. HPLC profile of pimobendan and its metabolite. The incubation was carried out in the presence (B) or the absence (A) of pimobendan for 15 min. The concentrations of pimobendan and microsomal protein used were 0.5 μM and 0.5 mg/ml, respectively. The activity of pimobendan *O*-demethylase was 21 pmol/mg/min.

3. Results and discussion

Typical chromatograms of samples of incubation mixtures with and without pimobendan are shown in Fig. 2. The peak of the *O*-demethylated metabolite of pimobendan showed baseline separation from the peak of pimobendan. No interfering peak was found in the chromatogram of an incubation mixture without pimobendan. The fluorescence intensity of the metabolite was increased about 360% by the addition of a mixture of methanol and phosphoric acid to the column effluent as described in Section 2.5. The retention times for pimobendan and its metabolite were 5.6 and 2.8 min, respectively. The pimobedan metabolite formed in vitro incubation of pimobendan with liver microsomes had the same retention time as *O*-demethylated pimobendan. The increase in peak area due to *O*-demethylated pimobendan was shown to be linear in the range of 0.1 to 200 ng (12 points), and the equation of the calibration curve was $y=5018x$ (y: peak area, x: ng metabolite). The intra- and inter-assay precision studies, performed at the substrate concentrations of 1 μM and 500 μM, showed good reproducibility, and the intra- and inter-assay relative standard deviations (RSDs) were 2.0%, 6.8%, 2.1% and 5.6%, respectively (Table 1). Furthermore, when the accuracy was calculated for the standard solution (50 μg/ml) used calibration curve (three points), the accuracy of the assay was 5.7%. The detection limit of the *O*-demethylated metabolite was 0.1 ng. The rate of formation of the *O*-demethylated metabolite from pimobendan was linear for up to 45 min when the concentrations of pimobendan and microsomal protein used in the

Table 1
Intra- and inter-assay variability of pimobendan *O*-demethylase activities

Substrate concentration (μM)	n	Variability (RSD, %)	
		Intra-assay	Inter-assay
1	6	2.0	6.8
500	6	2.1	5.6

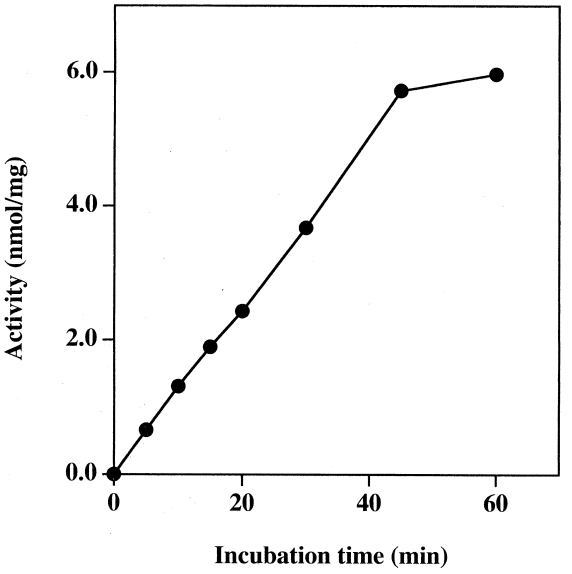

Fig. 3. Time course of pimobendan *O*-demethylation in rat liver microsomes. The activity of pimobendan *O*-demethylase was measured in the presence of 250 μ*M* substrate and 0.5 mg microsomal protein. Each data point represents the mean of duplicate determinations.

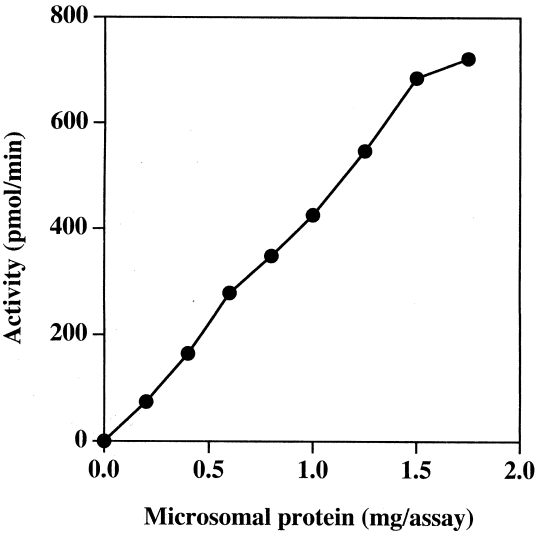

Fig. 4. Microsomal protein dependency of pimobendan *O*-demethylation. The *O*-demethylated metabolite formed was measured in the presence of various amounts of microsomal protein as specified in the figure. The concentration of pimobendan used was 250 μ*M*. The mixture was incubated for 15 min. Each data point represents the mean of duplicate determinations.

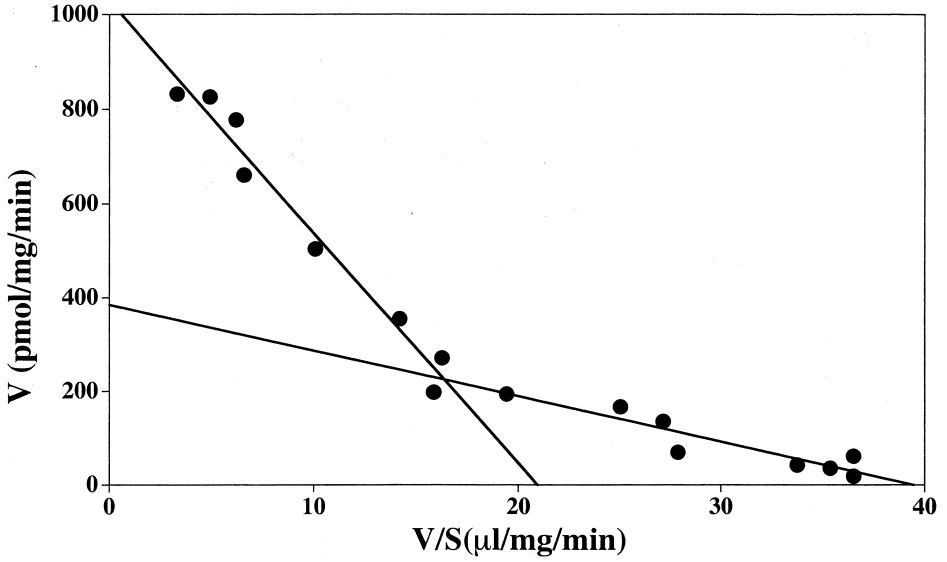

Fig. 5. Kinetics of *O*-demethylation of pimobendan in rat liver microsomes. The concentration range of substrate used for kinetic analysis was 0.5 μ*M* to 250 μ*M*. The *O*-demethylation of pimobendan was measured as described in Materials and Methods in the presence of 0.5 mg of microsomal protein. The data are plotted in the form of an Eadie–Hofstee plot.

incubation mixture were 250 μ*M* and 0.5 mg/ml, respectively (Fig. 3). In addition, the formation rate of the metabolite increased linearly with increasing concentration of microsomal protein up to 1.5 mg/ml (Fig. 4). Furthermore, the HPLC elution profile indicated that *O*-demethylated pimobendan is a major metabolite of pimobendan in rat liver microsomes.

A kinetic analysis of pimobendan *O*-demethylation was conducted using the method developed in the present study. As shown in Fig. 5, the pimobendan *O*-demethylation in rat liver microsomes was well described by a two-site kinetic model, indicating that at least more than one enzyme may be involved.

The method presented in this study is basically the same as previous method reported by Pahernik et al. [11] with regards to the detection techniques, but the analysis time of this method is substantially shorter than that of previous method. In addition, in view of its simplicity and sensitivity, we consider that the method described here is suitable for detailed kinetic studies of pimobendan metabolism in vitro.

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