

# Determination of zolmitriptan enantiomers in rat liver microsomes by chiral high performance liquid chromatography with fluorescence detection

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Received 31 December 2003; revised 30 May 2004; accepted 26 June 2004

**ABSTRACT:** A selective chiral high performance liquid chromatographic method was developed and validated to separate and quantify the enantiomers of a new potent selective 5-HT<sub>1B/1D</sub> receptor partial agonist, *S*-zolmitriptan, and its antipode in rat liver microsomes induced with  $\beta$ -naphthoflavone. *S*- and *R*-zolmitriptan were extracted from rat hepatic microsomal incubates with chloroform/isopropanol (75:25, v/v), and were separated on a narrow-bore enantioselective normal phase Chiralpak AD-H column (250  $\times$  0.46 mm) with hexane-isopropanol-triethylamine (72/28/0.25, v/v/v) as mobile phase and fluorescence detection with emission at 350 nm and excitation at 291 nm. The calibration curves were linear for *R*- and *S*-zolmitriptan concentration over the range 0.1–5.0  $\mu$ g/mL ( $r = 0.9996$  and  $0.9999$ ), and the limits of quantitation were 0.1  $\mu$ g/mL. The metabolism and interaction of the enantiomers of zolmitriptan in treated hepatic microsomes were investigated using chiral HPLC. There was significant difference between the disposition of the *S*- and *R*-zolmitriptan when racemic zolmitriptan or single enantiomers of zolmitriptan were incubated for 5, 10 and 20 min, suggesting that the metabolism of zolmitriptan in rat liver microsomes is enantioselective. In addition, there was also a significant difference between the IC<sub>50</sub> of *R*- to *S*-zolmitriptan and *S*- to *R*-zolmitriptan (IC<sub>50S/R</sub>/IC<sub>50R/S</sub> = 45.2). This indicated that the disposition process favored the *S*-form of zolmitriptan. Copyright © 2004 John Wiley & Sons, Ltd.

**KEYWORDS:** enantioselective; zolmitriptan; chiralpak AD-H; metabolism; interaction

## INTRODUCTION

Zolmitriptan (311C90) is a new potent selective 5-HT<sub>1B/1D</sub> receptor partial agonist (Martin, 1994; Martin *et al.*, 1997) that has recently been approved for use in the acute treatment of migraine and related vascular headaches. The main metabolic pathway of zolmitriptan involves CYP1A2 with isolated human hepatocytes (Wild *et al.*, 1999) and rat hepatic microsomes (Yu *et al.*, 2003). There are three major metabolites of zolmitriptan, one of which, the *N*-demethyl metabolite, shows 5-HT<sub>1D</sub> receptor agonist activity in isolated human hepatocytes (Wild *et al.*, 1999), but only one

metabolite (*N*-demethyl metabolite) was founded in rat hepatic microsomes (Yu *et al.*, 2003).

Differences in the clinical effect of many enantiomers are well known, such as ofloxacin (Fujimoto and Mitsuhashi, 1990), propranolol (Li and Zeng, 2000) and ibuprofen (Davies, 1998). Therefore, today regulatory authorities in the USA, Europe, China and Japan provide guidelines indicating that preferably only the active enantiomer (eutomer) of a chiral drug should be brought to the market. Zolmitriptan is one of those drugs, its *S*-enantiomer being used clinically. There are, however, few reports studying the other isomer of zolmitriptan (*R*-antipode), e.g. its pharmacodynamics, pharmacokinetics and metabolism. In particular, it is difficult to get rid of *R*-enantiomer absolutely in zolmitriptan, and the *R*-zolmitriptan remaining as an impurity may alter the pharmacodynamics, pharmacokinetics or disposition of *S*-zolmitriptan and lead to changes in its clinical effect.

It was reported that zolmitriptan enantiomers were separated by capillary zone electrophoresis (Yang *et al.*, 2003), but this has not been done in biological samples. In order to explore the metabolism of *R*-zolmitriptan and the possible interactions between the two enantiomers *in vitro*, a chiral chromatographic method using

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**Abbreviations used:** IC<sub>50</sub>, 50% inhibitory concentration; LOD, limit of detection; LOQ, limit of quantification; NADP,  $\beta$ -nicotinamide adenine dinucleotide phosphate; ODS, octadecylsilane.

Contract/grant sponsor: Natural Science Foundation of China; Contract/grant number: 30225047; 39770868.

Contract/grant sponsor: Zhejiang Provincial Natural Science Foundation; Contract/grant number: C397500; RC97016.

chiral stationary phase was validated to separate and assay two zolmitriptan enantiomers simultaneously in rat hepatic microsomes in our study. The metabolism and interaction of the enantiomers of zolmitriptan in treated hepatic microsomes were investigated using the chiral HPLC method developed.

## EXPERIMENTAL

**Chemicals and reagents.** The racemic zolmitriptan and its two optical isomers (purity >98%, HPLC) were kindly given by the Department of Chemistry (Zhejiang University, Hangzhou, China). Diphenyltriazol (internal standard) was a generous gift from Department of Pharmaceutical Chemistry (Zhejiang University, Hangzhou, China).  $\beta$ -Naphthoflavone (BNF), trinitric isocitric acid, isocitric dehydrogenase,  $\beta$ -nicotinamide adenine dinucleotide phosphate (NADP) and its reduced form (NADPH) were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals and solvents were analytical reagent or HPLC grade and obtained from common commercial sources.

The rat hepatic microsomes were prepared according to the method of Zhou *et al.* (2001) from rats (Sprague–Dawley, male, 170–210 g, age 6–7 weeks), which were pretreated with BNF (i.p. 80 mg/kg, 3 days), and immediately stored at  $-80^{\circ}\text{C}$ . Protein and cytochrome P450 contents were estimated according to the methods of Omura and Sato (1964).

**Incubation procedure.** Each incubation sample was performed with a 0.5 mL incubation mixture consisted of different concentration of substrate, *S*-zolmitriptan, *R*-zolmitriptan and racemic zolmitriptan, with rat liver microsomes (the final concentration was 0.75 mg protein/mL) and other cofactors (0.1 M Tris–HCl buffer, pH 7.4, 15 mM  $\text{MgCl}_2$ , 12 mM DL-isocitrate trisodium and 0.38 unit isocitrate dehydrogenase), respectively. After preincubating for 5 min at  $37^{\circ}\text{C}$ , 5  $\mu\text{L}$  solution of the regeneration system (0.172 M NADP and 0.048 M NADPH in 0.12 M  $\text{NaHCO}_3$ ) was added to each reaction mixture. After incubating at  $37^{\circ}\text{C}$  for indicating time, 2.0 mL of chloroform–isopropanol (75:25, v/v) were added followed by vortex mixing to stop the reaction. Then 5  $\mu\text{L}$  of concentrated ammonia water and 5  $\mu\text{L}$  of 0.1 mg/mL diphenyltriazol solution as internal standard were added, vortex mixed for 2 min, and centrifuged at 3500 rpm for 15 min. After that, the chloroform/isopropanol layer was separated and evaporated to dryness under a gentle stream of air at  $50^{\circ}\text{C}$ . The residue was reconstituted in 200  $\mu\text{L}$  of mobile phase, and 20  $\mu\text{L}$  was injected into HPLC system.

**Apparatus and chiral HPLC conditions.** The HPLC system (Shimadzu, Kyoto, Japan) consisted of two LC-10AD pumps, an SPD-10AV fluorescence detector, a CTO-10A column oven and N2000 data system (Zhejiang University, China). The samples were separated on a Chiralpak AD-H column (250  $\times$  4.6 mm, Daicel chemical Ind. Ltd) with a mobile phase containing hexane–isopropanol–triethylamine (72:28:0.25, v/v/v) at a flow rate of 0.8 mL/min at  $30^{\circ}\text{C}$ . The injected volume of the sample was 20  $\mu\text{L}$ . The excitation wavelength was set at 291 nm and the fluorescence was monitored at 350 nm.

The LC-MS system for the identification of metabolite of zolmitriptan consisted of an HP1100 series with a mass spectrometer with an electrospray ionization (ESI) source (Agilent technologies). The chromatographic separation condition was same as the above HPLC system, but the flow rate of mobile phase was set at 0.4 mL/min. The MS was operated in positive ESI mode. The ion mass spectra were scanned from 200 to 350. Single ion monitoring was accomplished by detecting *R/S*-zolmitriptan ( $m/z$  288) and *N*-demethylzolmitriptan ( $m/z$  274).

**Time-dependent experiment.** Racemic zolmitriptan, 2.0  $\mu\text{g}/\text{mL}$ , or 1.0  $\mu\text{g}/\text{mL}$  of single enantiomer of zolmitriptan used as substrate was added to rat liver microsomal incubation and the resulting mixture was preincubated at  $37^{\circ}\text{C}$  for 5 min. Then 5  $\mu\text{L}$  solution of regeneration system were added to each reaction mixture. After incubated at  $37^{\circ}\text{C}$  for 5, 10 and 20 min, 2.0 mL of chloroform–isopropanol (75:25, v/v) was added followed by vortex mixing to stop the reaction. The following steps were carried out according to the method as described under Incubation Procedure.

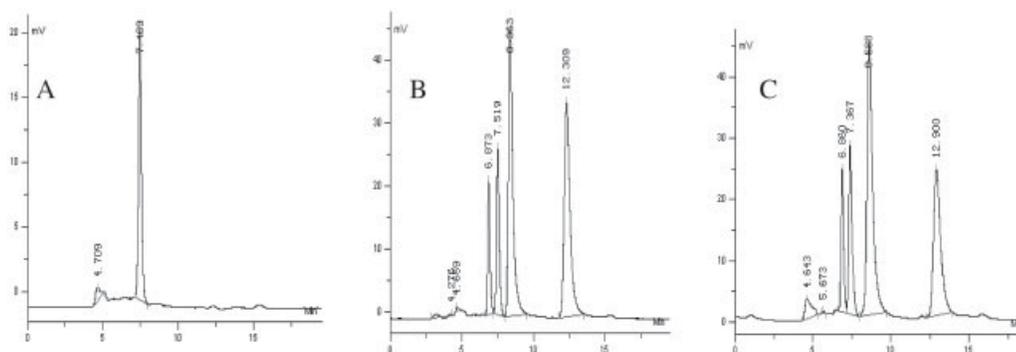
**Interaction study.** Single zolmitriptan enantiomer, 0.8  $\mu\text{g}/\text{mL}$ , and cofactors with different concentrations of its antipode were added into rat liver microsomes (0.75 mg protein/mL). To study the  $\text{IC}_{50}$  of *R*-zolmitriptan inhibiting *S*-zolmitriptan, *R*-zolmitriptan concentrations were set from 0 to 40  $\mu\text{g}/\text{mL}$ . For study of the  $\text{IC}_{50}$  of *S*-zolmitriptan inhibiting *R*-zolmitriptan, *S*-zolmitriptan concentrations were set from 0 to 2.0  $\mu\text{g}/\text{mL}$ . The resulting mixtures were preincubated at  $37^{\circ}\text{C}$  for 5 min. Then 5  $\mu\text{L}$  solution of regeneration system was added to each reaction mixture and incubated for 10 min. The amount of remaining zolmitriptan was determined by the same method as described above. Apparent  $\text{IC}_{50}$  values were determined by nonlinear regression analysis of the data (SAS statistical analysis system).

**Data analysis.** All data are presented as mean  $\pm$  SD. Groups of data were compared by one-way analysis of variance and, if significant, were further evaluated by Student's *t*-test (SAS statistical analysis system). Differences were considered significant when the *p*-value was  $<0.05$ , unless otherwise specified.

## RESULTS AND DISCUSSION

### Chromatographic separation

Figure 1 shows the chromatograms obtained from (A) blank sample, (B) blank microsomal incubation contained racemic zolmitriptan and diphenyltriazol (internal standard), (C) sample after incubated for 5 min. This HPLC system is specific for simultaneous determination of zolmitriptan and internal standard in rat hepatic microsomes. The peaks were sharp indicating high column efficiencies for two enantiomers and internal standard. Zolmitriptan ( $m/z$  288) and its metabolite of demethylzolmitriptan ( $m/z$  274) were detected using HPLC-MS. The metabolites did not interfere with the determination of zolmitriptan.



**Figure 1.** Chromatograms of extracted rat hepatic microsomal incubate. (A) Blank sample; (B) blank microsomal incubate with racemic zolmitriptan and diphenytriazol (internal standard); (C) sample after incubating for 5 min. The peak at 6.8 min is the internal standard, the peak at 9.6 min *R*-zolmitriptan and the peak at about 12.3 min *S*-zolmitriptan. Chiralpak AD-H column; mobile phase hexane/isopropanol/triethylamine (72:28:0.25, v/v/v); flow rate, 0.8 mL/min; fluorescence detection,  $\lambda_{\text{ex}} = 291 \text{ nm}$ ,  $\lambda_{\text{em}} = 350 \text{ nm}$ .

**Table 1.** Recovery and precision for assay of *R*- and *S*-zolmitriptan in rat hepatic microsomes incubates (mean  $\pm$  SD,  $n = 5$ )

Concentration spiked ( $\mu\text{g/mL}$ )	Recovery (%)		Relative standard deviations (%)			
			Intra-day		Inter-day	
	<i>R</i> -	<i>S</i> -	<i>R</i> -	<i>S</i> -	<i>R</i> -	<i>S</i> -
0.2	107.2 $\pm$ 7.2	108.2 $\pm$ 3.3	7.4	3.3	5.6	7.0
1.0	95.2 $\pm$ 1.6	97.9 $\pm$ 3.5	1.6	3.5	4.0	5.2
5.0	100.2 $\pm$ 6.1	100.1 $\pm$ 6.8	6.1	6.8	6.3	3.5

### Linearity and the limit of quantification

The calibration curves for the sample assay as per the procedure above were found to be linear over the concentration range 0.1–5.0  $\mu\text{g/mL}$  for both *R*- and *S*-zolmitriptan with 0.5 mL incubate solution. Peak area ratios ( $y$ ) of the *R*- and *S*-zolmitriptan vs the internal standard were measured and plotted against the concentration ( $x$ ) of *R*- or *S*-zolmitriptan. The regression equations of the calibration curves of *R*- and *S*-zolmitriptan were  $y = 3.4232x + 0.2294$  ( $r = 0.9996$ ) and  $y = 3.4181x - 0.0684$  ( $r = 0.9999$ ). The lowest concentration of these calibration curves, which also represents the limit of quantification (LOQ), was 0.1  $\mu\text{g/mL}$  with RSD of 14.8% ( $n = 5$ ) for *R*-zolmitriptan and 13.0% ( $n = 5$ ) for *S*-zolmitriptan. The limit of detection (LOD) of *R*- and *S*-zolmitriptan was 0.02  $\mu\text{g/mL}$  ( $S/N > 3$ ).

### Precision and accuracy

The precision and accuracy of the method were examined by adding known amounts of *R*- and *S*-zolmitriptan to pooled rat hepatic microsomes (blank control samples). For intra-day precision and accuracy five replicate quality control samples at each concentration were assayed on the same day. The inter-day precision

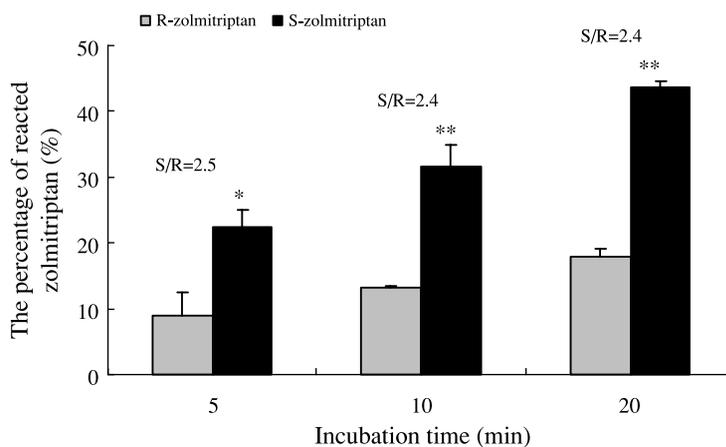
and accuracy were evaluated on five different days. The obtained results are summarized in Table 1. The intra-day and inter-day precisions were within 10%. The criteria of both precision and accuracy for analyzing biological samples were fulfilled in the developed method.

### Recovery

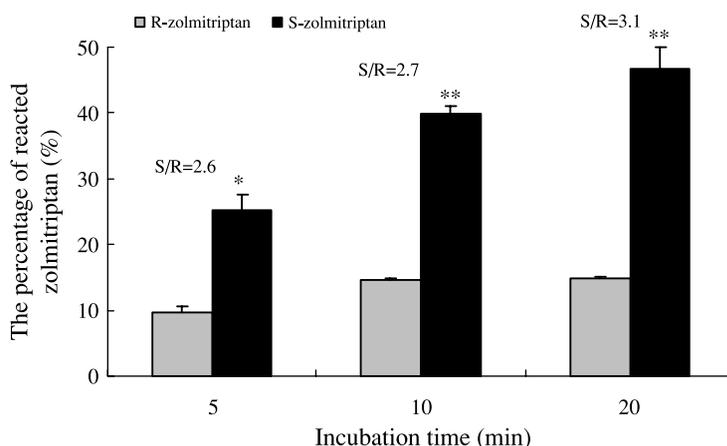
Chloroform–isopropanol (75:25, v/v) was used as the extraction solvent, which was based on our previous method (Yu *et al.*, 2003). The internal standard, diphenytriazol, also had good recovery in this condition. The peak area ratios of *R*- and *S*-zolmitriptan and internal standard were compared with calibration curve of *R*- and *S*-zolmitriptan, respectively. The recoveries of *R*/*S*-zolmitriptan were summarized in Table 1. The average recovery of this analytical method was 101.5%.

### Time-dependent metabolism and interaction study

After the single zolmitriptan enantiomer was incubated for 5, 10 and 20 min in rat liver microsomes incubate, the percentage of reacted *R*- and *S*-zolmitriptan was significantly different, and the ratio of the percentage of



**Figure 2.** Difference between the metabolism of *R*- and *S*-zolmitriptan in rat liver microsomes after incubating for 5, 10 and 20 min. Data are expressed as means  $\pm$  SD of three independent experiments. *S/R* represents the ratio of percentage of reacted *S*- to *R*-zolmitriptan. \* Significant difference ( $p < 0.05$ ); \*\* more significant difference.

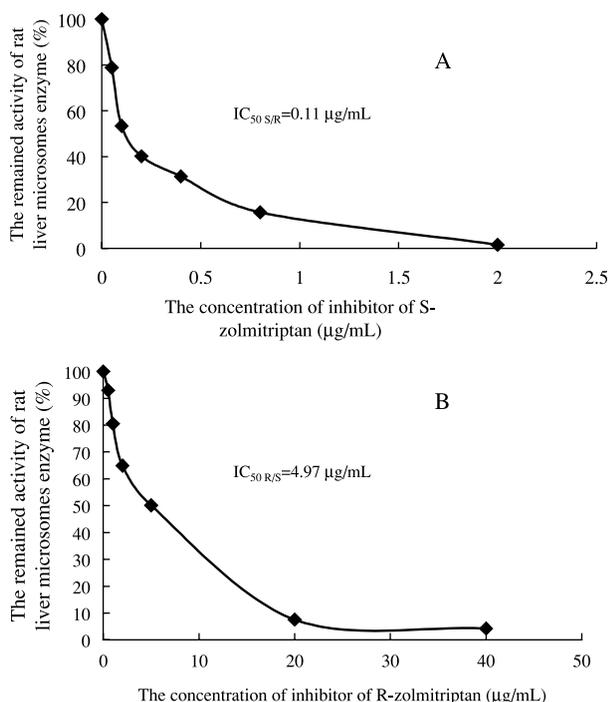


**Figure 3.** Difference of the metabolism of *R*- and *S*-zolmitriptan in rat liver microsomes after racemic zolmitriptan was incubated for 5, 10 and 20 min. Data are expressed as means  $\pm$  SD of three independent experiments. *S/R* represents the ratio of percentage of reacted *S*- to *R*-zolmitriptan. \* Significant difference ( $p < 0.05$ ); \*\* more significant difference ( $p < 0.01$ ).

reacted *S*-zolmitriptan to that of *R*-zolmitriptan (*S/R*) at the indicated time was about 2.4 (Fig. 2). This indicated that the disposition velocity of *S*-zolmitriptan was faster than that of *R*-zolmitriptan in rat liver microsomes. When the racemic zolmitriptan was incubated in rat liver microsomes, the ratio of percentage of reacted *S*-zolmitriptan to *R*-zolmitriptan (*S/R*) at the indicated time was 2.6, 2.7 and 3.1, respectively (Fig. 3). This means that there is a disposition interaction effect between the two enantiomers.

Zolmitriptan is used clinically as the single enantiomer, *S*-zolmitriptan. There was, however, little information on the reason for this. Interestingly, Seaber *et al.* (1996) reported that the metabolite, *N*-demethylated

metabolite, showed 5-HT<sub>1D</sub> receptor agonist activity and was found in human plasma at concentrations likely to contribute to the therapeutic activity of the compound. Although the results obtained using rat liver microsomes cannot replace studies using human hepatic microsomes, rat liver microsomes, pretreated with specific inducers, provide sound models to study metabolism of xenobiotics *in vitro* (Nebbia *et al.*, 1999; Sarver *et al.*, 1998; Yamazaki *et al.*, 2001; Soucek, 1999; Easterbrook *et al.*, 2001). The disposition process favored the *S*-form of zolmitriptan in induced rat hepatic microsomes in our study. The result showed that this is, maybe, one reason why zolmitriptan is used clinically as *S*-zolmitriptan.



**Figure 4.** Inhibition curves for the interactions between *S*- and *R*-zolmitriptan in rat liver microsomes *in vitro*. (A) The  $IC_{50}$  of *S*-zolmitriptan to the metabolism of *R*-zolmitriptan; and (B) the  $IC_{50}$  of *R*-zolmitriptan to the metabolism of *S*-zolmitriptan.  $IC_{50}$  values were determined by nonlinear regression.

There was also a significant difference ( $p < 0.001$ ) between the  $IC_{50R/S}$  for *R*-inhibiting *S*-zolmitriptan and the  $IC_{50S/R}$  for *S*-inhibiting *R*-zolmitriptan (Fig. 4). The  $IC_{50S/R}$  was  $0.11 \pm 0.01 \mu\text{g/mL}$ , which was much lower than that of  $IC_{50R/S}$  ( $4.97 \pm 0.23 \mu\text{g/mL}$ ). This indicated that *S*-zolmitriptan is a potent metabolism inhibitor to *R*-zolmitriptan ( $IC_{50S/R}/IC_{50R/S} = 45.2$ ), but the inhibited effect of *R*-zolmitriptan on *S*-zolmitriptan is not significant in rat liver microsomes. That indicates that the *R*-zolmitriptan remaining as an impurity in zolmitriptan does not significantly alter the disposition of *S*-zolmitriptan.

## CONCLUSION

The chiral HPLC method described in this investigation was suitable for the study of the stereoselective metabolism and interaction of zolmitriptan in rat liver microsomes. Stereoselective disposition of zolmitriptan was observed, and it was found the disposition process favored the *S*-form of zolmitriptan.

## Acknowledgement

This project was supported by Natural Science Foundation of China (30225047 and 39770868) and Zhejiang Provincial Natural Science Foundation of China (C397500 and RC97016).

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