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*SHORT COMMUNICATION*

# Stereoselective determination of propafenone enantiomers in transgenic Chinese hamster CHL cells expressing human cytochrome P450

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ABSTRACT: An enantioselective assay for *S*(+)- and *R*(−)-propafenone in transgenic Chinese hamster CHL cells expressing human cytochrome P450 was developed. The method involved extraction of propafenone from the S9s incubates, and formation of propafenone diastereomeric derivatives with the chiral reagent 2,3,4,6-tetra-*O*-*β*-D-glucopranosyl isothiocyanate. Separation and quantitation of diastereomeric propafenone derivatives were carried out in a reverse-phase-HPLC system with UV detection. The assay was linear from 2 to 200 μg/mL for each enantiomer. The analytical method gave average recoveries of 97.5% and 97.0% for *S*(+)- and *R*(−)-propafenone, respectively. The limits of detection and quantitation for the method are 0.1 and 2.0 μg/mL for both *S*(+)- and *R*(−)-propafenone, respectively. The reproducibility of the assay was good (RSD <10%). The method allowed study of the depletion of *S*(+)- and *R*(−)-propafenone in transgenic Chinese hamster CHL cells expressing human cytochrome P450. The stereoselectivity of propafenone phase I metabolism via cDNA-expressed CYP3A4 was observed. Copyright © 2000 John Wiley & Sons, Ltd.

## INTRODUCTION

Propafenone, 1-[2-[2-hydroxy-3-(propylamino)-propoxy]phenyl]-3-phenyl-1-propanone, is a widely used antiarrhythmic agent administrated as the racemic form. The two enantiomers, *S*(+)- and *R*(−)-propafenone, are equipotent in terms of sodium channel-blocking activity, but the main side effect, ie *β*-adrenoreceptor-blocking action, resides in the *S*(+)-isomer. The stereoselective disposition of racemic propafenone has been reported in humans (Kroemer *et al.*, 1989). However, so far the phase I metabolism of racemic propafenone *in vitro* has not been studied using chiral chromatography. Several analytical methods for determining the enantiomers of propafenone in biological fluids have been reported, including the isotopic label method (Kroemer *et al.*, 1990), or the chiral stationary phase (Bohm *et al.*, 1995), chiral mobile phase addictive (Kern, 1994), or chiral derivatization method with (*R*)-1-(2-nathylethyl isothiocyanate (Mehvar, 1990). In this article a reliable enantioselective analytical method for assay of *S*(+)- and *R*(−)-propafenone in transgenic Chinese hamster CHL cells expressing human cytochrome P450 was developed by using reverse-phase (RP)-HPLC with *S*-propranolol as internal standard. The method reported in this paper has been applied to study the stereoselectivity of phase I metabolism of propafenone enantiomers *in vitro*.

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## EXPERIMENTAL

**Materials.** Racemic propafenone hydrochloride (99.5%) was obtained from Yiatai Pharmaceutical Company (Zhejiang, China). NADPH (98%), S(−)-propranolol, S(+)-propafenone, R(−)-propafenone and 2,3,4,6-tetra-*O*-*β*-D-glucopranosyl isothiocyanate (GITC) were purchased from Sigma (St Louis, MO, USA). All other chemicals and solvents were of analytical or chromatographic grade. Transgenic Chinese hamster CHL cells expressing specific human live cytochrome P450 were provided by the Department of Pathology and Physiology (College of Medicinal Sciences, Zhejiang University, China).

S9s were prepared according to the following procedure. Cells grown confluence in 25 $cm^2$ tissue culture flasks were washed twice with phosphate-buffered saline (PBS, pH 7.4), and harvested by scraping. Subsequently the cells were sonicated for $10 \times 5$ s with 10 s interval in 0.15 mol/L KCl at 4°C. The mixture solution were centrifugated at 9000 ***g*** for 20 min at 4°C. The supernatant, ie S9s, was stored at −70°C.

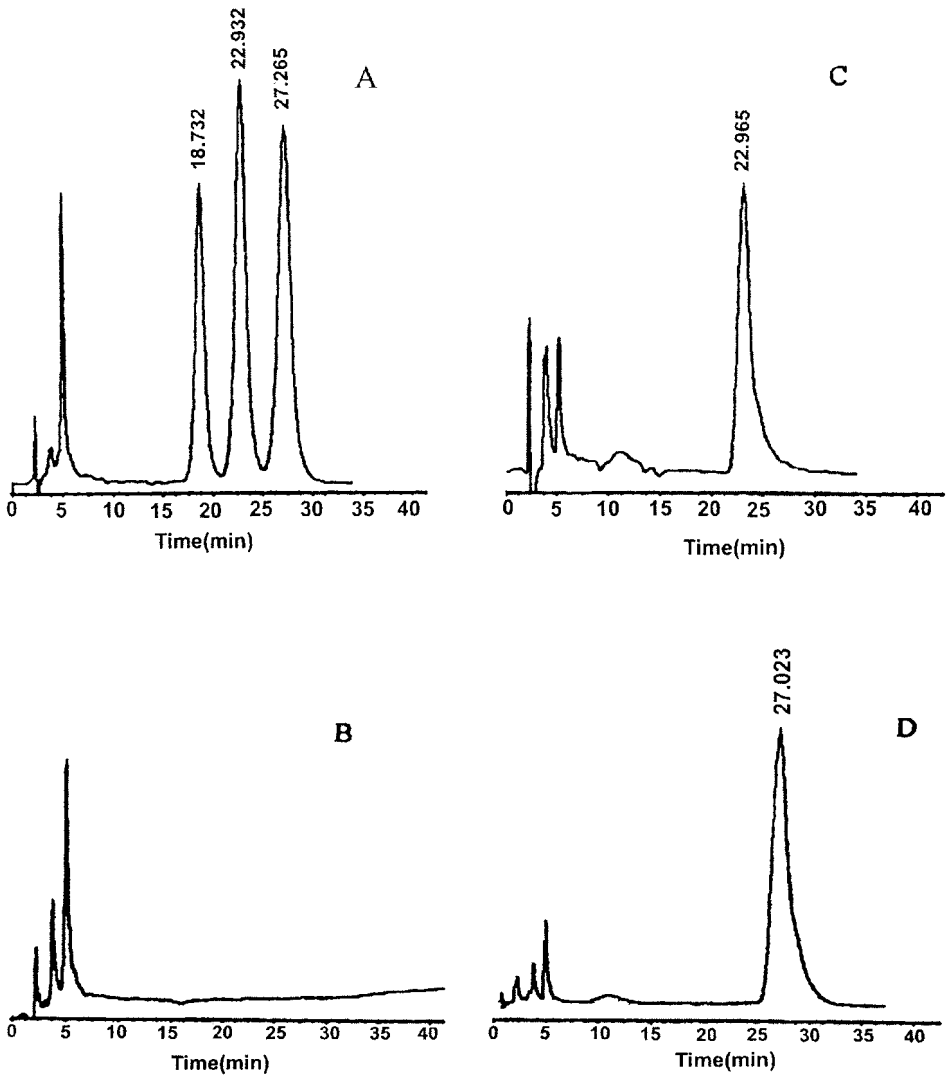


**Figure 1.** Chromatograms of propafenone and internal standard after pre-colum dervatization. (A) Blank S9s incubates spiked with propafenone and internal standard [$t_R$ = 18.7 min for *S*(−)-propranolol, 22.9 min for *S*(+)-propafenone, and 27.2 min for *R*(−)-propafenone]. (B) Blank S9s incubate. (C, D) Blank S9s incubates spiked with *S*(+)-propafenone and *R*(−)-propafenone, respectively.

**Instrumentation.** The modular HPLC equipment was an LC-10AT VP pump with a SPD-10A VP detector (Simadzu, Japan) and upper data system (Zhejiang University, China). The analytical column was a Shim pack CLC ODS (150 × 4.6 mm i.d., 5μm). The mobile phase was a mixture of methanol–water–glacial acetic acid (67:33:0.05, v/v/v) with a flow rate of 0.8 mL/min. The wavelength of UV detector was set at 220 nm.

**Assay procedure.** The pH of 250 μL of incubation media (0.6 mg/mL protein and cofactors) was adjusted to 10 by adding 200 μL of 25% ammonia solution. *S*(−)-propranolol (30 μL of 250 μg/mL in methanol) was added as an internal standard. Propafenone was extracted with 1.0 mL chloroform by vortex for 3 min. The chloroform layer was separated by centrifugation at 4000 rpm for 10 min, then evaporated to dryness under a gentle stream of air at 45°C. The residue was dissolved in 100 μL of GITC solution (4 mg/mL in acetonitrile), and allowed to stand at 35°C for 30 min. The reaction mixture was evaporated to dryness under a gentle air stream. The residue was reconstituted with 200 μL mobile phase and aliquot of 20 μL of the resulting solution was injected into the HPLC system.

**Incubation of propafenone with S9s prepared from transgenic Chinese hamster CHL cells expressing cytochrome P450 3A4 (CYP3A4).** The time-dependent study was performed with a 250 μL incubation mixture containing 5 μg/mL *S*(+)- or *R*(−)-propafenone with 0.15 mg protein equivalent of S9s prepared from transgenic Chinese hamster CHL cells expressing CYP3A4. The incubation mixture was bubbled with oxygen for 1 min before use. After preincubation in water bath at 37°C for 5 min, 30 μL NADPH solution (50 mg/mL in pH 7.4 buffer) was added to initiate the reaction. The reaction was stopped by adding

 

**Table 1. Metabolic depletion of *S*(+)- and *R*(−)-propafenone ($\bar{x} \pm s$, $n = 3$)**

| Incubation time (min) | Amount remaining (μg/mL) | | *S/R* ratio | *V* (μg/min) | |
|---|---|---|---|---|---|
| | *S*(+)-propafenone | *R*(−)-propafenone | | *S*(+)-propafenone | *R*(−)-propafenone |
| 0 | 5.000 | 5.000 | 1.00 | | |
| 1 | 3.978 ± 0.05 | 3.879 ± 0.06 | 1.03 | 0.256 | 0.280 |
| 2 | 3.499 ± 0.05 | 3.053 ± 0.05 | 1.15 | 0.188 | 0.243 |
| 5 | 3.026 ± 0.06 | 2.640 ± 0.03 | 1.15 | 0.099 | 0.118 |
| 15 | 2.924 ± 0.21 | 2.522 ± 0.17 | 1.16 | 0.035 | 0.041 |

1.0 mL chloroform at the incubation time of 0, 1, 2, 5 and 15 min. The internal standard was added and the enantiomers in the samples were measured according to the Assay procedure section, above.

## RESULTS

### Chromatographic specificity

The chromatogram of S9s incubate, spiked with racemic propafenone and *S*(−)-propranolol, showed a good separation and high column efficiency for the three compounds (Fig. 1). The α and resolution between *S*(−)-propranolol and *S*(+)-propafenone were 1.25 and 1.60, respectively. The α and resolution between *S*(+)- and *R*(−)-propafenone were 1.21 and 1.56, respectively. The peaks of *S*(−)-propranolol and *S*(+)-, *R*(−)-propafenone were verified with pure enantiomer standards. A blank S9s incubate was carried out according to the Assay procedure section, above. No interference appeared at the peak positions of *S*(−)-propranolol and *S*(+)-, *R*(−)-propafenone.

### Calibration curves and sensitivity of the assay

Calibration curves for *S*(+)- and *R*(−)-propafenone were contructed by analyzing a serials of blank S9s incubates spiked with racemic propafenone in the enantiomeric concentration range from 2 to 200 μg/mL. Extraction and derivatization of the samples were carried out as described in the Assay procedure action. Peak area ratios ($y$) of the *S*(+)- or *R*(−)-enantiomers to the internal standard were measured and plotted against the concentrations ($x$) of each enantiomer. The linearity of the calibration curves for *S*(+)- and *R*(−)-propafenone was in the range from 2.0 to 200.0 μg/mL. The regression equations of the calibration curves were $y = 0.01617x - 0.02565$ ($r = 0.9994$) for *S*(+)-propafenone and $y = 0.01656x - 0.02432$ ($r = 0.9994$) for *R*(−)-propafenone.

The LOD (limit of detection) for each enantiomer was 100 ng/mL and the LOQ (limit of quantitation) was 2.0 μg/mL (RSD < 10%, $n = 5$).

### Recovery studies

A serials of blank S9s incubates, spiked with different concentrations (5 and 200 μg/mL) of *S*(+)- or *R*(−)-propafenone, were processed as described under *Assay procedure*. The peak area ratios of enantiomer to internal standard were compared with those obtained by analyzing aqueous samples containing equal amounts of propafenone. The average recovery of this analytical method was 97.5 ± 1.5% and 97.0 ± 1.8% ($n = 5$) for *S*(+)- and *R*(−)-propafenone, respectively.

### Reproducibility studies

The drug-free incubation media, spiked with different concentrations (5 and 200 μg/mL) of *S*(+)- or *R*(−)-propafenone, were used for reproducibility studies. The intra-assay variability was determined by analyzing samples in quintuplicate, and inter-assay variability by analyzing samples in quintuplicate on five separate days, according to the procedure described under Assay procedure. The relative standard deviations were calculated.

The within-day and between-day relative standard deviations (RSD) for the assay of enantiomers in S9s incubates were both less than 10%. This showed that the reproducibility and repeatability of the enantioselective HPLC method for *S*(+)- and *R*(−)-propafenone in S9s incubates was satisfactory.

### Phase I: metabolism of propafenone enantiomers

The metabolic depletion of *S*(+)- and *R*(−)-propafenone at various incubation times and the metabolic rate of *S*(+)- and *R*(−)-propafenone are shown in Table 1. The rate of *R*(−)-propafenone metabolism is faster than that of the *S*(+)-antipode. The *S/R*-propafenone ratio is from 1.03 to 1.16 when the time of metabolic reaction was from 1 to 15 min. So far as we are aware, that this is the first report on the stereoselective metabolism of racemic propafenone via CYP3A4. The results show that the phase I metabolism of propafenone enantiomers at 5 μg/mL via CYP3A4 is enantioselective.

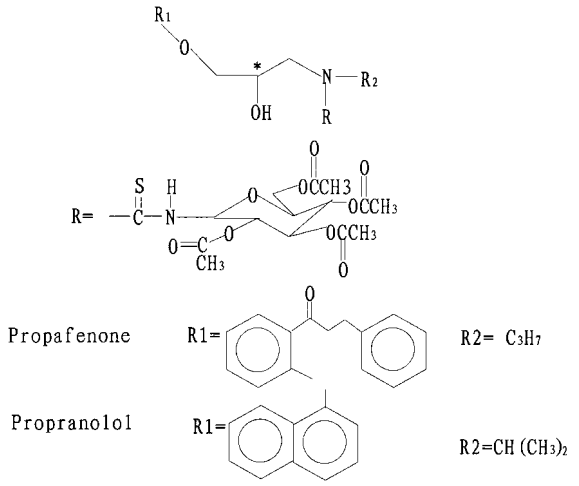


**Figure 2.** Structures of propafenone, internal standard and their GITC derivatives.

## DISCUSSION

In chiral drug metabolism study, many factors are involved, such as extraction or derivatization, so it is necessary to use internal standard. In this assay, *S*(−)-propranolol was really a boon (Fig. 2). Racemic propranolol and metoprolol were also tested as candidates for internal standard, but the derivative of *R*(+)-propranolol overlapped with that of *R*(−)-propafenone, and metoprolol enantiomers were eluted too early at 9 and 13 min.

The absolute recovery of *S*(+)- or *R*(−)-propafenone from the samples alkalized with ammonia solution was almost the same as with those without alkalinization, but the recovery of *S*(−)-propranolol was very low without alkalinization. When NaOH was used as alkalizer, the diastereomers in the mobile phase were unstable. Five interfering peaks, which decreased the column efficiency, with retention times from 9.9 to 15.0 min were observed in HPLC chromatograms. Morever, the GITC derivatives were unstable. The peak area of diastereomer of *S*(−)-propranolol decreased by 38%, *S*(+)-propafenone 39% and *R*(−)-propafenone 17% in 35 min, which resulted in decreasing the *S/R* ratio of propafenone significantly. Where ammonia solution was used as an alkalizer, the derivatives were stable for at least 1 week.

Chloroform can form an azeotropic mixture with water, so the sample extracted with chloroform did not need extra dryness in this assay. Methylene chloride should not be used because trace impurities in it can easily react with GITC and cause interferences. Ether can also be used as an extraction solvent, with a recovery slightly less than chloroform. In addition, chloroform was able to precipitate protein in incubates and stop the enzymic reaction. Triethylamine was observed to have the capability to catalyze the derivatization of propafenone. The amount of GITC added to each sample was sufficient to assure the completion of derivatization. The large excess of GITC did not spoil the chromatogram. Good reproductibility was achieved if derivatization occurred at 35°C for 30 min.

## CONCLUSION

A specific stereoselective analytical method was developed for the assay of *S*(+)- and *R*(−)-propafenone in S9s incubates. The method was applied to the study of *S*(+)- and *R*(−)-propafenone depletion *in vitro*. The results show that propafenone undergoes enantioselective metabolism at 5 μg/mL concentration via cDNA-expressed CYP3A4.

### Acknowledgements

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