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High-performance liquid chromatographic assay of (±)-ethopropazine and its enantiomers in rat plasma[1]

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Abstract

Two high-performance liquid chromatographic (HPLC) methods are described for determination of (±)-ethopropazine (ET) in rat plasma. After deproteination and liquid–liquid extraction, assay of (±)-ET was performed using either a C_{18} column (non-stereospecific assay) or an (α-*R*-naphthyl)ethylurea column (stereospecific assay). The UV detection was at 250 nm. Mean recovery was >85%. Both assays demonstrated excellent linear relationships between peak height ratios and plasma concentrations; quantitation limits were ≤25 ng/ml, based on 100 μl rat plasma. Accuracy and precision were <17% with both methods. Both methods were applied successfully to the measurement of ET plasma concentrations in rats given the drug intravenously.

Keywords: Enantiomer separation; Ethopropazine

1. Introduction

Ethopropazine (Fig. 1), also known as profenamine (10-(2-diethylaminopropyl) phenothiazine HCl; Parsitan, Parsidol), is an anticholinergic drug which is used to treat patients afflicted with either Parkinson's disease or drug-induced extrapyramidal symptoms [1,2]. Chemically, ET shares some structural similarities to other anticholinergic drugs used in Parkinson's disease (e.g. trihexyphenidyl, procyclidine, and orphenadrine). For instance, ET contains a highly lipophilic aromatic moiety and an alkyl side chain containing a chiral center and tertiary amine functional group (Fig. 1).

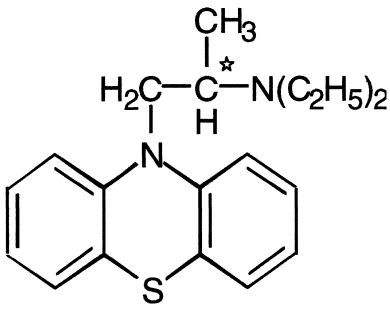

Fig. 1. Structure of ethopropazine; chiral center denoted by asterisk.

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Similar to other anticholinergic agents used in Parkinson's disease, there is little available information which describes the pharmacokinetics of ET in humans or animals. This is perhaps surprising, considering that the drug has been in clinical use for more than 40 years [3,4]. This may be attributed to factors such as the discovery of levodopa for Parkinson's in the 1960's, which has resulted in a decreased sense of importance and interest in the use of the anticholinergic drugs, patent expiry issues, or perhaps due to the lack of analytical methods sensitive and specific enough for quantitation of ET in biological specimens. Whatever the reason, the anticholinergic drugs, including ET, are still used clinically, and an understanding of the pharmacokinetic properties of drugs is important in their use.

In order to shed light on the pharmacokinetics of ET, we have developed rapid, sensitive and specific HPLC methods for measurement of ET and its enantiomers in rat plasma. In this report we describe the assay methods and their utility in performing preliminary pharmacokinetic studies in the rat.

2. Experimental

2.1. Materials and chemicals

(±)-Ethopropazine–HCl, (±)-orphenadrine–HCl and (±)-diphenidol–HCl were obtained from Sigma (St. Louis, MO, USA). Methanol, acetonitrile, hexane, methylene chloride, absolute ethanol (all HPLC grade) and trifluoracetic acid, triethylamine and sulfuric acid were purchased from EM Science (Gibbstaun, NJ, USA). Potassium phosphate (monobasic) and sodium phosphate (mono and dibasic) were obtained from BDH (Toronto, ON, Canada).

2.2. Chromatographic conditions

Two HPLC systems were used for analysis. The first consisted of a Waters Model 600 E pump (Waters, Milford, MA, USA), Beckman 507e autosampler and a Beckman 166 variable wavelength UV absorbance detector (Beckman, Mississauga, ON, Canada). Data collection, integration and calibration were accomplished using Beckman System Nouveau computer software (Mississauga, ON, Canada). The second HPLC system consisted of a Waters Model 510 pump, a Waters WISP 710 B autosampler and Waters Model 481 variable wavelength UV absorbance detector (Waters, Milford, MA, USA). Integration was performed using a Shimadzu integrator Model C-R5A (Shimadzu, Kyoto, Japan).

For non-stereospecific analysis, the chromatographic separations of ET and internal standard (orphenadrine) were accomplished using a 4.6×150 mm Beckman Ultrasphere ODS column (Mississauga, ON, Canada). The mobile phase consisted of methanol; acetonitrile; potassium phosphate (0.025 *M*); (25:25:50, v/v) plus 0.75 ml sulfuric acid (2 *M*) and 0.25 ml triethylamine per l of mobile phase.

For stereospecific analysis, we adopted the chromatographic conditions developed by Ponder et al. [5]. That procedure utilized a stainless steel analytical column (100×4.6 mm I.D.) packed with 5 μm (α-*R*-naphthyl)ethylurea (YMC ARNU; YMC, Wilmington, NC, USA). The mobile phase was a combination of hexane, dichloromethane, ethanol and trifluoracetic acid (58:25:17:0.17, v/v).

For both assays, isocratic conditions (1 ml/min), room temperature and UV detection at 250 nm were used. This wavelength represented the UV maximum, of a solution of ET in methanol. The UV maxima were 250 nm and 252 nm in the mobile phases utilized in the non-stereospecific and stereospecific analyses, respectively.

For measuring the optical activity of ET enantiomers, a Perkin-Elmer 241 polarimeter (Norwalk, CT, USA) was used. The volume of the measuring cell was 1 ml and the length of the optical path was 10 cm.

2.3. Standard and stock solutions

A stock drug solution was prepared by dissolving 10.8 mg of (±)-ET–HCl in 100 ml of methanol (100 μg/ml of (±)-ET base). This solution was stored at −20°C between use; ET remained stable in solution under these conditions for at least 6 months. The working standard solutions were prepared daily from the stock solution by sequential dilution with sodium phosphate buffer (pH=5.9) to yield final concentrations of 10, 1 and 0.1 μg/ml of (±)-ET.

The internal standard stock solutions were prepared by dissolving 11.0 and 13.0 mg of orphenadrine–HCl (non-stereospecific assay) and diphenidol

HCl (used in stereospecific assay) respectively, in 100 ml of methanol. These two solutions were stored at 4°C between use.

2.4. Extraction procedure

The same extraction procedure was used for both the stereo- and non-stereospecific methods. In a 1.5-ml polypropylene centrifuge tube, 100 μl of rat plasma was added along with internal standard solution (50 μl and 60 μl for the nonstereo- and stereospecific assays, respectively). Plasma proteins were precipitated by addition of 0.3 ml acetonitrile while the tubes were vortex mixed. The tubes were subsequently centrifuged for 2 min and the supernatant was carefully transferred to new glass tubes using Pasteur pipets. To each tube, 0.3 ml of phosphate buffer (pH=5.9) and 3 ml of hexane were added. The tubes were then vortex mixed for 45 s and centrifuged at 2500×*g* for 3 min. The organic solvent layer was transferred to new tubes and evaporated to dryness under nitrogen. The residues were reconstituted using 180 μl of mobile phase and aliquots of 60–120 μl were injected onto the column.

2.5. Recovery

The extraction efficiency was determined by comparing peak heights from drug - free plasma (0.1 ml) spiked with known amounts of racemic ET to peak heights of the same amounts of (±)-ET which were directly injected onto the column without extraction. Recovery was determined at 25 ng/ml (n=8), 250 ng/ml (n=8) and 2500 ng/ml (n=4) of (±)-ET, using non-stereospecific chromatography. The recoveries of both internal standards (orphenadrine and diphenidol) were also evaluated using the same amounts employed in each assay procedure.

2.6. Calibration, accuracy and precision

Quantification was based on calibration curves constructed using peak height ratios of drug to internal standard, vs. nominal drug concentration. Intra-day reproducibility for the non-stereospecific method was tested by using four different concentrations per day in quadruplicate (12.5, 25, 125 and 500 ng/ml). The procedure was repeated on three separate days to allow for determination of inter-day precision and accuracy. For the stereospecific method, intra-day accuracy and precision was determined. Four concentrations of ET enantiomers (25, 50, 125, 500 ng/ml of each enantiomer) were assayed in quadruplicate. Intra-day accuracy was estimated based on the mean percentage error, and the inter-day accuracy was calculated as the mean of the intra-day accuracy determinations. The precision, expressed as a percentage, was evaluated by calculating the intra- and inter-day relative standard deviations.

2.7. Determination of optical activity of enantiomers

In order to establish the optical activity of ET enantiomers, HPLC eluent fractions corresponding to the two enantiomers were collected and subjected to polarimetry. To 10 mg of (±)-ET–HCl, 1 ml HPLC grade water, 250 μl of sulfuric acid (2 *M*) and 1.2 ml of sodium hydroxide (1 *M*) were added. Then, ET was twice extracted using 5 ml of hexane. After addition of hexane the tubes were vortex mixed, centrifuged at 2500×*g* for 3 min, and the organic layers were transferred and evaporated to dryness under nitrogen. The residue was reconstituted with 4 ml of mobile phase and aliquots of 20 μl were assayed using stereospecific HPLC chromatographic conditions. As the peaks corresponding to the two enantiomers eluted from the YMC ARNU column, the eluent fractions were collected manually in test tubes. After approximately 90 injections were made into the HPLC the eluent fractions were pooled, then concentrated to dryness in vacuo. Subsequently the residues were dissolved in 2 ml of methanol. To measure the purity and the concentrations of the collected enantiomers, each solution was assayed using stereospecific chromatography. These two solutions were used for the determination of optical rotation.

2.8. Racemization

The occurrence of racemization during assay and storage of samples was assessed using the eluent fractions containing isolated ET enantiomers. To check enantiomer stability during sample workup, approximately 25% of the eluent fractions described

above were taken and dried under nitrogen. Three ml of hexane were added, and 20 μl was directly placed into an autosampler vial containing 150 μl of mobile phase. Another 20 μl aliquot of the hexane solution was transferred to a clean tube and dried under nitrogen. After addition of 100 μl rat plasma, the sample was extracted as described above. Both samples were then subjected to stereospecific HPLC. To assess for the degree of racemization, the peak height ratio of (+):(−) enantiomers in the extracted sample was compared to that of unextracted sample.

To determine enantiomer stability during storage in plasma, eluent from peak 1 (10 μl) was added to test tubes and dried under nitrogen. Rat plasma (100 μl) was then added. Four samples were left 16 h at room temperature, and then assayed using the stereospecific method. Another four samples were frozen at −20°C for 7 days, then assayed using stereospecific chromatography.

2.9. Animal study

The assay procedures were tested for utility in preliminary pharmacokinetic studies in two male Sprague-Dawley rats (~300 g each). Under anesthesia with halothane administered using an anaesthetic machine, a silastic cannula was implanted into the right jugular vein of each animal. Each rat received a single dose of ET solution (60:30:10, v/v of water; propylene glycol USP; absolute ethanol) of either 10 or 20 mg/kg over 1 min, via the cannula. Serial blood samples were collected from the cannula for up to 72 h, and the resultant plasma samples were kept at −20°C until assayed. The area under the plasma concentration vs. time curve (AUC) was determined using the log-linear trapezoidal rule. The estimated initial plasma concentration immediately after dosing was determined by fitting compartmental models to the concentration vs. time data using the computer program SAAM II (SAAM Institute, Seattle, WA, USA).

3. Results

The mean extraction efficiency for (±)-ET from 100 μl of rat plasma at concentrations of 25, 250, and 2500 ng/ml were 90.8%, 88.3%, and 85.8%, respectively. Mean recoveries of the internal standards, orphenadrine and diphenidol, were 67.4% and 76.9%, respectively. In the non-stereospecific chromatographic method, peaks corresponding to internal standard, (±)-orphenadrine and (±)-ET eluted free of interfering substances, at 5.7 and 7.5 min, respectively (Fig. 2A). Excellent linear relationships ($r^2 \geq$ 0.997) were obtained between peak height ratios and the corresponding plasma concentrations over a range of 12.5 to 3000 ng/ml of (±)-ET; the mean regression line from the validation runs was described by (±)-ET (ng/ml)=(peak height ratio− 0.0126)÷0.00184. The intra-day and inter-day coefficients of variation were less than 10% over a wide range of (±)-ET concentrations (Table 1). The validated lower limit of quantitation of this assay was 12.5 ng/ml (Table 1).

Under the chromatographic conditions for stereospecific analysis, the internal standard, (±)-diphenidol, eluted as a single peak at 6.6 min, and the ET enantiomers eluted at approximately 8.3 and 9.4 min. After separation of ET enantiomers using fraction collection, 1.87 and 1.65 mg of purified peak 1 and peak 2 were recovered, respectively. The enantiospecific purities of peak 1 and peak 2 were 99.6% and 92.5% respectively. Re-assay of the enantiomers from rat plasma spiked with the eluent fractions indicated that there was no racemization of ET during the extraction procedure. There was also no evidence of racemization after storage overnight at room temperature, or after storage for 1 week at −20°C. Upon subjecting the fractions to polarimetry, the enantiomer corresponding to peak 1 gave a negative optical rotation, whereas peak 2 gave a positive value. These findings were consistently replicated over a number of wavelengths. Thus, peak 1 was assigned a levo- or (−) optical rotation, and peak 2 was assigned the dextro- or (+) descriptor.

Using stereospecific chromatography, all peaks were free of interference from endogenous components, and the peaks corresponding to ET enantiomers were baseline resolved (Fig. 2B). Linear responses were observed in the analyte/IS peak height ratio for enantiomer concentration from the range of 25 to 500 ng/ml. the correlation coefficient for calibration curves were equal to 0.998 for each enantiomer. The regression lines were best described by (−)-ET (ng/ml)=(peak height ratio +0.00339)÷

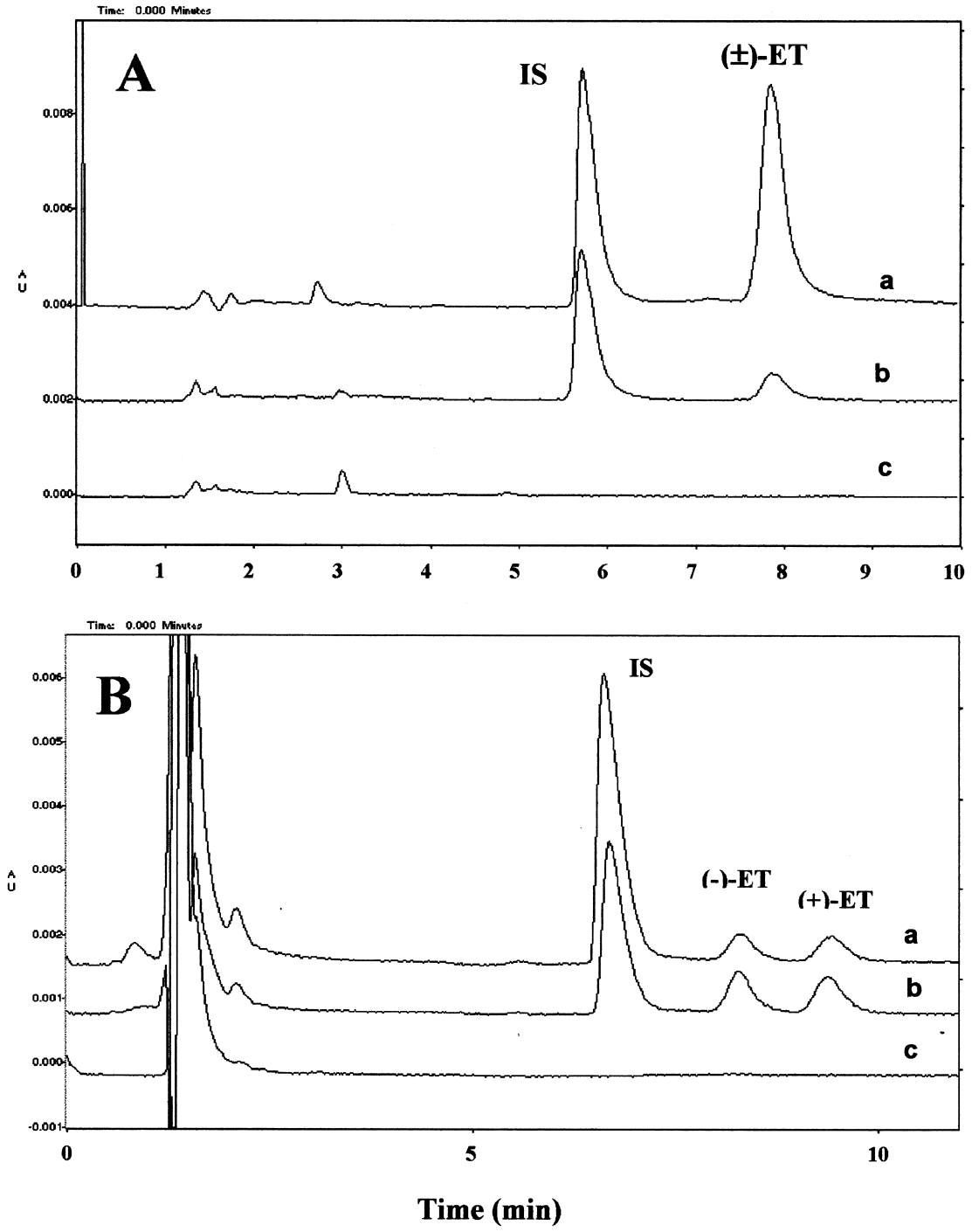

Fig. 2. (A) Chromatograms, obtained using ODS column, of (a) plasma sample taken from a rat 12 h after an IV dose of 20 mg/kg (±)-ET–HCl, (b) rat plasma spiked with 125 ng/ml of (±)-ET, (c) drug free rat plasma. (B) Chromatograms, obtained using (α-*R*-naphthyl)ethylurea column, of (a) plasma sample obtained 12 h after an IV dose of 10 mg/kg (±)-ET–HCl, (b) rat plasma spiked with 125 ng/ml of each ET enantiomer, (c) drug free rat plasma.

Table 1
Accuracy and precision of non-stereospecific HPLC method

Nominal spiked concentration (ng/ml)	Intra-day measured concentration, mean±SD (ng/ml)			Inter-day measured concentration, mean±SD (ng/ml)	Average inter-day accuracy (%)	Average inter-day precision (%)
	Run 1 (n=4)	Run 2 (n=4)	Run 3 (n=4)			
12.5	11.2±0.97	13.1±1.56	11.8±0.77	12.1±1.10	96.4	9.37
25	21.4±3.09	22.9±1.17	24.8±1.45	23.0±1.90	92.1	7.40
125	122±4.45	124±10.5	141±7.74	129±7.60	103	8.17
500	517±14.8	515±11.8	531±44.2	521±23.6	104	1.64

Table 2
intra-day accuracy and precision of stereospecific HPLC method

Nominal enantiomer concentration (ng/ml)	Measured enantiomer concentration, mean±SD (ng/ml)		Average precision (%)		Average accuracy (%)	
	(−)-ET	(+)-ET	(−)-ET	(+)-ET	(−)-ET	(+)-ET
25	29.2±1.26	25.8±1.63	4.32	6.31	117	103
50	52.6±1.00	50.6±2.02	1.89	3.99	105	101
125	122±0.75	114±2.34	0.61	2.05	97.8	91.1
500	497±11.6	459±10.4	2.34	2.26	99.4	91.9

0.000727 and (+)-ET (ng/ml)=(peak height ratio+ 0.00235)÷0.000659. The validated limit of quantitation was 25 ng/ml for each enantiomer. The C.V.% was less than 7% and bias less than 17% (Table 2).

In the rat given a 20 mg/kg dose of (±)-ET–HCl, plasma samples were analyzed using the non-stereospecific method (Fig. 3A). The area under the plasma concentration–time curve (AUC_{0-24h}) was 12.0 mg·h/l. Plasma samples from the second rat, which was given 10 mg/kg of IV (±)-ET–HCl, were assayed using stereospecific chromatography (Fig. 3B). The AUC_{0-24h} for (−)- and (+)-ET were 4.87 and 4.78

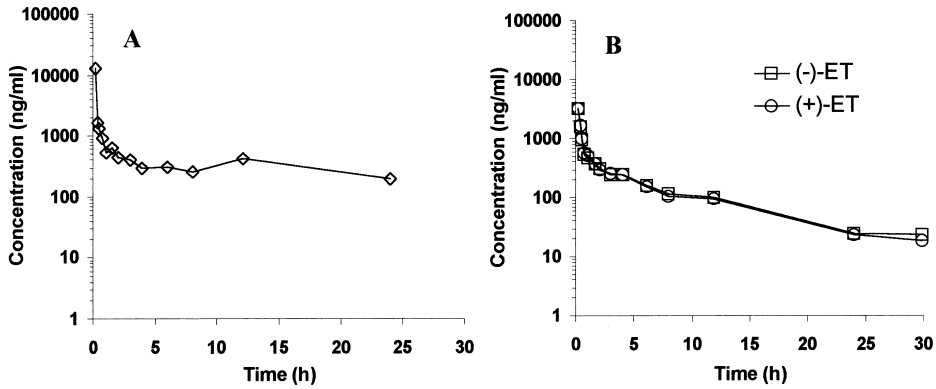

Fig. 3. (A) (±)-Ethopropazine plasma vs. time profile from a rat given 20 mg/kg IV; samples assayed by non-stereospecific HPLC. (B) Plasma vs. time profiles of ethopropazine enantiomers in a rat given 10 mg/kg IV; samples assayed using stereospecific HPLC.

mg h/l. Using SAAM II, a three compartment open model was best fit to the plasma concentration vs. time data.

4. Discussion

Here, we have presented accurate, precise and sensitive HPLC methods for the determination of ET in plasma, suitable for use in pharmacokinetic studies in the rat. Other investigators have described analytical methods for the determination of ET [6–9]. However only one of these methods was applied to use in a biological matrix [7]. Ishikawa et al. [7] used solid-phase extraction and GC–MS chromatography/detection to qualitatively separate ET from other phenothiazine drugs in urine and plasma. Although extraction of 20 μg/ml of (±)-ET was reported to be 66% from 1 ml of human plasma or urine, validation data including lower limit of quantitation was not reported.

Both HPLC methods performed well with respect to reproducibility and accuracy over the range of concentrations studied (Tables 1 and 2). The chromatographic conditions employed by Ponder et al. [5] for the separation of ET enantiomers worked well in conjugation with our extraction method (Fig. 2). The lower limits of quantitation for both assays were ideal for studying the stereo- or nonstereoselective pharmacokinetics pharmacokinetics of ET in the rat. Both assay methods were rapid; preparation of 20 samples took less than 2 h from protein precipitation to placement of samples in the HPLC autosampler vials. For both assays, chromatographic run times were less than 10 min.

In the rat given 10 mg/kg of (±)-ET there was no observed stereoselectivity in the plasma concentrations of the enantiomers. In this rat, it took 30 h for plasma concentrations of both enantiomers to fall below the lower limit of detection (25 ng/ml each enantiomer). Blood samples were only collected for 24 h in the rat given 20 mg/kg, so we could not assess the duration of time concentrations stayed above the lower limit of quantitation of 12.5 ng/ml (±)-ET.

In conclusion the described methods were found to be suitable for the analysis of ET in samples collected during pharmacokinetic studies in rats. Although this is the first pharmacokinetic data reported for ET, only two rats were studied. More study is required to definitively characterize the pharmacokinetics of the drug. The lack of stereoselectivity in the plasma concentrations of ET after IV injection of racemate suggests that the non-stereospecific assay is appropriate for use in pharmacokinetic studies in the rat.

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