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# Direct analysis of the enantiomers of mexiletine and its metabolites in plasma and urine using an HPLC–CSP*

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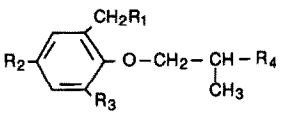
**Abstract**: A high-performance liquid chromatographic assay has been developed for the quantification of the enantiomers of mexiletine and its four major metabolites, in plasma and in urine. Mexiletine and all metabolites were determined, after derivatization of mexiletine and its hydroxymetabolites, *p*-hydroxymexiletine and hydroxymethylmexiletine, using a Chiralpak AD chiral stationary phase, based on a carbamoyl derivative of amylose. *o*-phthalaldehyde was chosen as derivatization reagent to increase the sensitivity of detection, to achieve separation of all compounds in one chromatographic system, and to avoid interferences.

**Keywords**: *Enantioselective HPLC; p-hydroxymexitletine; hydroxymethylmexiletine; p-hydroxymexiletine-alcohol; hydroxymethylmexiletine-alcohol.*

## Introduction

Mexiletine, 1-(2,6-dimethylphenoxy)-2-aminopropane (MEX, Fig. 1), is an oral antiarrhythmic drug which is similar to lidocaine in structure and physiological effects [1]. It has been shown to be effective in the treatment of neuropathic pain of different etiologies [2–4]. MEX is a chiral molecule and is clinically administered as a racemic mixture. The enantiomers of MEX differ in their pharmacokinetic disposition [5–7], binding to cardiac sodium channels [8], and electrophysiological effects [9].

MEX is extensively metabolized in humans, and the major metabolites are hydroxymethylmexiletine (OH-ME-MEX), *p*-hydroxymexiletine (*p*-OH-MEX) and their corresponding alcohols, hydroxymethylmexiletine-alcohol (OH-ME-MEX-OL) and *p*-hydroxymexiletine-alcohol (*p*-OH-MEX-OL) (Fig. 1) [10]. In serum samples the hydroxymetabolites and OH-ME-MEX-OL were found, but not *p*-OH-MEX-OL [11]. *In vitro* tests with human liver microsomes and the single enantiomers showed that hydroxylation reactions, catalysed by the P450IID6, an isoenzyme of P450, exhibit stereoselectivity [12].




| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| MEX | H | H | $CH_3$ | $NH_2$ |
| OH-ME-MEX | OH | H | $CH_3$ | $NH_2$ |
| p-OH-MEX | H | OH | $CH_3$ | $NH_2$ |
| OH-ME-MEX-OL | OH | H | $CH_3$ | OH |
| p-OH-MEX-OL | H | OH | $CH_3$ | OH |
| KOE 0768 (intemal std) | H | $CH_3$ | H | $NH_2$ |

**Figure 1**
The structures of MEX and its metabolites.

Several methods have been published for the indirect analysis of MEX enantiomers as diastereomeric derivatives by GC [5] and by reversed-phase HPLC [13, 14]. A direct method for the enantioselective separation of MEX enantiomers has been reported using a Pirkle-type chiral stationary phase [6]. However, to our knowledge, a method for the determination of the enantiomers of MEX and its four major metabolites in biological fluids has not been published.

This manuscript reports the development of an enantioselective HPLC assay for the determination of the enantiomers of MEX and its metabolites, in total 10 compounds, in plasma and urine. This approach utilizes a derivatiz-

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ation of MEX and its hydroxymetabolites with *o*-phthalaldehyde which was initially used by Gupta and Lew for the achiral determination of MEX [15]. In this method, all the target compounds could be separated in one chromatographic system using a Chiralpak AD chiral stationary phase (AD-CSP). The derivatized and non-derivatized compounds were monitored at different wavelengths under slightly modified mobile phase conditions, and at different temperatures.

The method has been validated for use in a study of MEX in the treatment of cancer related neuropathic pain. The goal of this study is the investigation of the relationship between pain relief and plasma levels of MEX and its metabolites. If effective plasma concentrations can be established, individually adjusted dosages can be used to maximize efficacy and minimize toxicity.

## Experimental

### *Chemicals*

Racemic mexiletine, *S*-(+)-mexiletine, *R*-(−)-mexiletine and *p*-hydroxymexiletine as hydrochloride salts, hydroxymethylmexiletine oxalate, *p*-hydroxymexiletine-alcohol, hydroxymethylmexiletine-alcohol and the internal standard 1-(2,4-dimethylphenoxy)-2-aminopropane (KOE 0768) as hydrochloride were kindly provided by Boehringer Ingelheim (Burlington, Canada). HPLC-grade ethanol and hexane were purchased from Anachemia (Montreal, Canada). Sodium carbonate monohydrate and sodium hydrogen carbonate were obtained from BDH (Toronto, Canada).

### *Apparatus*

The chromatographic system consisted of a P2000 pump, a AS880 autosampler, a UV1000 and a FL2000 detector (all Spectraphysics, San Jose, CA, USA), respectively. Data collection was carried out using a ChromJet Integrator (Spectraphysics) connected to a Spectra 386 compatible computer running WINner 386 autolab software (Spectraphysics). A Haake D1 thermostatic waterbath (Haake, Karlsruhe, Germany) was used for temperature control of the chiral stationary phase.

MEX separations were obtained on columns packed with chiral stationary phases (CSP) based on $\alpha_1$-acid glycoprotein (Chiral-AGP, 100 mm × 4 mm, Regis Chemical Co., Morton Grove, IL, USA), ovomucoid (ULTRON ES-OVM, 150 mm × 4.6 mm, MAC-MOD Analytical, Chadds Ford, PA, USA) and cellulose tris(3,5-dimethylphenyl carbamate) (Chiralcel OD-H, 250 mm × 4.6 mm, Chiral Technologies, Exton, PA, USA).

Separations of MEX and its metabolites after derivatization were achieved on a CSP based on amylose tris(3,5-dimethylphenyl carbamate) (Chiralpak AD, 250 mm × 4.6 mm, Chiral Technologies). For plasma and urine samples a 5 μm nitrile guard cartridge (10 mm × 3 mm, Regis Chemical Co.) was placed in front of the Chiralpak AD-CSP.

### *Chromatographic conditions*

*Separations of the enantiomers of mexiletine.* (A) Chiral-AGP; the mobile phase consisted of 0.01 M sodium phosphate buffer (pH 7.5)–2-propanol (98:2, v/v) at a flow rate of 0.9 ml $min^{-1}$, UV-detection was at 216 nm.

(B) ULTRON ES-OVM; the mobile phase was 0.01 M, phosphate buffer (pH 7.0)–methanol (95:5, v/v) at a flow rate of 0.8 ml $min^{-1}$; UV-detection was at 216 nm.

(C) Chiralcel OD-H with phenyl guard cartridge; the mobile phase consisted of hexane–ethanol–diethylamine (98:2:0.5, v/v/v) at a flow rate of 1.0 ml $min^{-1}$; fluorescence detection at $\lambda_{ex}$ = 270 nm and $\lambda_{em}$ = 312 nm.

In all cases ambient temperature was used.

*Determination of MEX and its metabolites in plasma and urine.* (A) The separation of MEX, *p*-OH-MEX and OH-ME-MEX after derivatization was carried out using a mobile phase consisting of hexane–ethanol (94:6, v/v up to 15 min, 75:25, v/v from 15.1 min to the end of the run) at a flow rate of 1.0 ml $min^{-1}$; the column temperature was 20°C; fluorescence detection at $\lambda_{ex}$ = 350 nm and $\lambda_{em}$ = 444 nm.

(B) For the determination of the non-derivatized metabolites, OH-ME-MEX-OL and *p*-OH-MEX-OL, the mobile phase consisted of hexane–ethanol (90:10, v/v), at a flow rate of 0.8 ml $min^{-1}$; the column temperature was 0°C. The excitation and the emission wavelengths of the fluorescence detector were 270 and 312 nm, respectively.

### *Preparation of standards*

Stock solutions of racemic mexiletine, its metabolites and internal standard were prepared in methanol. The concentrations were 1 mg $ml^{-1}$ for mexiletine and 100 μg $ml^{-1}$ for the metabolites and the internal standard (all

as free base). Appropriate working dilutions of the stock solutions in methanol were made.

*Sample preparation*

*Derivatization of MEX, OH-ME-MEX and p-OH-MEX in plasma.* To 1 ml of plasma were added 100 μl of internal standard solution (10 μg $ml^{-1}$ in methanol KOE 0768, Fig. 1) and 1 ml 0.5 M sodium carbonate buffer (pH 10) followed by 7 ml of diethylether. The tubes were vortexed for 1 min; centrifuged at 1750*g* for 10 min; frozen in a slurry of dry ice and acetone; and the organic layer transferred to a clean tube. The diethylether was evaporated under nitrogen, the residue reconstituted in 200 μl of *o*-phthalaldehyde reagent [prepared daily by mixing three volumes of *o*-phthalaldehyde (1 mg $ml^{-1}$ methanol) with one volume of 2-mercaptoethanol (25 μl/25 ml methanol)] [15] and diluted with methanol (1:1). After 5 min the methanol was evaporated under nitrogen, the residue reconstituted in 100 μl of mobile phase and 50 μl injected onto the column.

*p-OH-MEX-OL and OH-ME-MEX-OL in plasma.* Diethylether (7 ml) was added to 1 ml of plasma, the resulting mixture was vortexed for 1 min; centrifuged at 1750*g* for 10 min; frozen in a slurry of dry ice and acetone; and the organic layer transferred to a clean tube. The diethylether was evaporated under nitrogen, the residue reconstituted in 100 μl of mobile phase and 50 μl injected onto the column.

*Derivatization of MEX, OH-ME-MEX and p-OH-MEX in urine.* To 100 μl of urine were added 200 μl of internal standard (KOE 0768; 10 μg $ml^{-1}$ in methanol), 500 μl of sodium carbonate buffer (0.5 M, pH 10) and 5 ml of diethylether. The samples were then processed using the procedures described above for plasma concentrations of these compounds.

*p-OH-MEX-OL and OH-ME-MEX-OL in urine.* Diethylether (5 ml) was added to 100 μl ml of urine, the resulting mixture was vortexed for 1 min; centrifuged at 1750*g* for 10 min; frozen in a slurry of dry ice and acetone; and the organic layer transferred to a clean tube. The diethylether was evaporated under nitrogen, the residue reconstituted in 100 μl of mobile phase and 50 μl injected onto the column.

*Test of the derivatization procedure*

The extent of derivatizations of MEX, OH-ME-MEX and *p*-OH-MEX and the internal standard were investigated as a function of *o*-phthalaldehyde derivatization reagent and reaction time. Standard dilutions containing MEX and its four metabolites at 0.1 and 1 μg, respectively, and 1 μg internal standard were evaporated under nitrogen and derivatized as described under 'sample preparation'. The reaction times were 1, 5, 30 and 120 min.

For the stability test of the derivatives, a standard dilution containing MEX and its metabolites was evaporated under nitrogen. The residue was reconstituted in *o*-phthalaldehyde reagent. After 5 min reaction time the methanol was evaporated under nitrogen, the residue reconstituted in 100 μl of mobile phase and 20 μl were injected onto the column.

To test the linearity of the derivatization, standard dilutions containing MEX, the metabolites and the internal standard were derivatized as described under sample preparation. The concentrations for MEX and the metabolites were 0.1, 0.2, 0.5, 1 and 2 μg and the concentration of the internal standard was 1 μg. In order to test the linearity of the derivatization with physiological ratios of mexiletine to its metabolites, a second experiment was conducted with MEX/metabolite concentrations of 1.2/0.2, 1.5/0.5 and 2/1 μg.

*Standard curves*

Standard curves were prepared by spiking drug-free plasma and urine with known amounts of mexiletine and its four metabolites prior to the extraction procedure described above. For the plasma assay the concentrations used were 0.02, 0.1, 0.5, 1, 2 μg $ml^{-1}$ for the derivatized compounds, and 0.02, 0.05, 0.1, 0.2, 0.5 μg $ml^{-1}$ for the underivatized compounds. For the urine assay the spike concentrations were 0.02, 0.1, 0.5, 1, 2 (additionally for mexiletine, 10), μg/100 μl for the derivatized compounds, and 0.02, 0.05, 0.1, 0.2, 0.5 μg $ml^{-1}$ for the underivatized compounds. The standard curves were run in duplicate.

## Results and Discussion

*Enantioselective resolutions of MEX*

Enantioselective resolutions of MEX were obtained on the protein-based CSPs Chiral-AGP and ULTRON ES-OVM (Table 1, Fig.

**Table 1**
Chromatographic parameters of mexiletine separation on chiral stationary phases (CSP)

| CSP | $k'_1$* | $\alpha$† | $R_s$‡ |
|---|---|---|---|
| Chiral-AGP | 13.6 | 1.1 | 1.6 |
| ULTRON-ES-OVM | 7.0 | 1.2 | 2.6 |
| Chiralcel OD-H | 2.9 | 1.1 | 1.2 |

For chromatographic conditions see text.
*Capacity factor of first-eluted enantiomer.
†Separation factor.
‡Stereochemical resolution.

2A,B). Close to baseline separations were achieved on the Chiral-AGP with mobile phases composed of phosphate buffer (0.01 M, pH 7.5) and a variety of uncharged modifiers including 2-propanol, ethanol, methanol and acetonitrile (2–5%). On the ULTRON ES-OVM, a baseline separation was observed for the MEX enantiomers. However, when the metabolites were chromatographed, only *p*-OH-MEX-OL was enantioselectively resolved on the Chiral-AGP. Mexiletine was also separated on a Chiralcel OD-H column (Table 1 and Fig. 2C).

*Enantioselective resolution of MEX and its metabolites in plasma and urine*

The enantioselective separation of the MEX metabolites but not of the parent compound was possible on a Chiralpak AD-CSP. However, OH-ME-MEX was not separated on this CSP at ambient temperatures and subambient chromatography at 0°C was used to enantioselectively resolve this compound.

In order to increase the sensitivity of the assay to determine plasma and urine concentrations of MEX, OH-ME-MEX and *p*-OH-MEX, the compounds were derivatized with the achiral fluorophore *o*-phthalaldehyde. An unexpected consequence of this derivatization was the enantioselective resolution of the MEX derivative on the Chiralpak AD-CSP which made it possible to separate all three compounds in a single run. The separations of the isoindole derivatives are presented in Fig. 3. *o*-phthalaldehyde reacts in the presence of thiols with primary amines to form fluorescent isoindole derivatives; therefore MEX and its hydroxymetabolites are derivatized but the alcohols, OH-ME-MEX-OL and *p*-OH-MEX-OL, are not.

Since OH-ME-MEX-OL and *p*-OH-MEX-OL are not derivatized and since their fluorophores differ from the isoindole derivatives, they do not interfere with the detection of MEX and the hydroxylated metabolites. OH-ME-MEX-OL and *p*-OH-MEX-OL are analysed separately from MEX and its hydroxylated metabolites using slightly different mobile phase conditions, different excitation and emission wavelengths and a different temperature, 0°C. The latter parameter re-

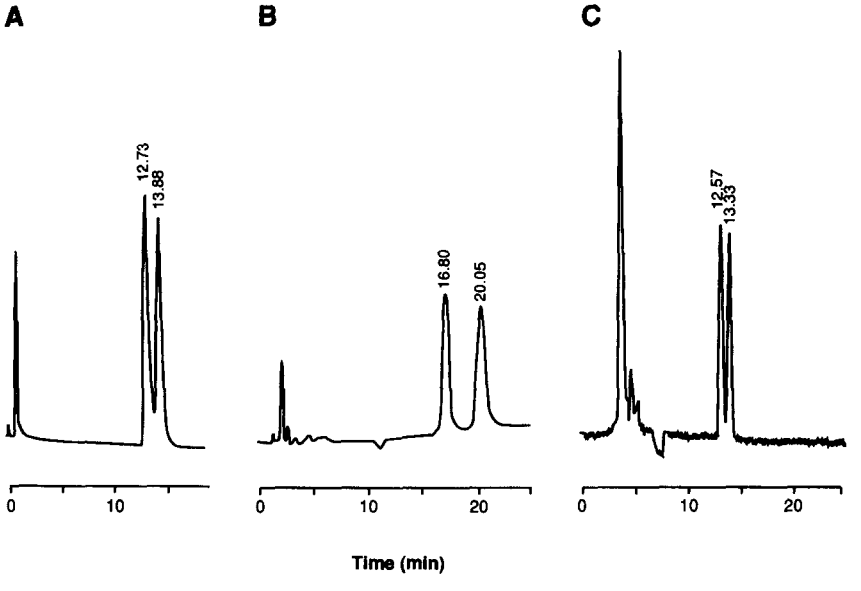


**Figure 2**
Chiral separations of MEX on different HPLC chiral stationary phases. (A) Chiral-AGP; (B) ULTRON ES-OVM; (C) Chiralcel OD-H. The enantiomeric elution orders are not indicated. See text for chromatographic conditions.

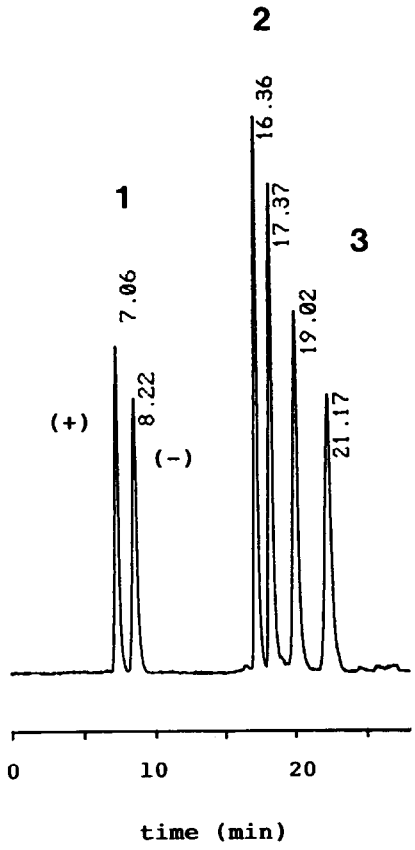


**Figure 3**
Separation of MEX, OH-ME-MEX, *p*-OH-MEX on a Chiralpak AD chiral stationary phase after pre-column derivatization. 1(+) = (*S*)-(+)-MEX; 1(−) = (*R*)-(−)-MEX; 2 = enantiomers of OH-ME-MEX; and 3 = enantiomers of *p*-OH-MEX. Chromatographic conditions: Chiralpak AD column with nitrile guard cartridge; mobile phase: *n*-hexane–2-propanol (90:10, v/v up to 10 min, from 11 min to the end of the run 75:25, v/v); flow rate 1.0 ml $min^{-1}$; detection: $\lambda_{ex}$ = 350 nm and $\lambda_{em}$ = 444 nm; ambient temperature.

flects the strong influence of temperature on the separation of the two alcohols which is illustrated in Fig. 4.

*Derivatization procedure*

A derivatization time of 5 min was found to be optimal. Longer reaction time did not improve the derivatization yield.

The stability test showed for eight samples, injected during 5 h, for the single enantiomers of mexiletine and its metabolites a relative standard deviation between 6.1 and 12.3%. After 24 h two more samples were injected showing 46.5–87.0% of the mean area of the first 5 h.

The calibration curves for the linearity test of the derivatives were linear for all compounds and the correlation coefficients were between 0.993 and 1.000. The test of standard mixtures with different higher amounts of mexiletine gave accuracies between 97.1 and 114.3%.

*Validation*

Calibration curves for the plasma and the urine assays for all the compounds were prepared in the range of 0.02–2 μg $ml^{-1}$ for the derivatized compounds, and 0.02–0.5 μg $ml^{-1}$ for the underivatized compounds. The calibration curves for all compounds were linear over the concentration ranges.

*Plasma and urine samples*

The application of this method to plasma and urine samples from a patient receiving an oral dose of 100 mg rac-MEX twice a day for

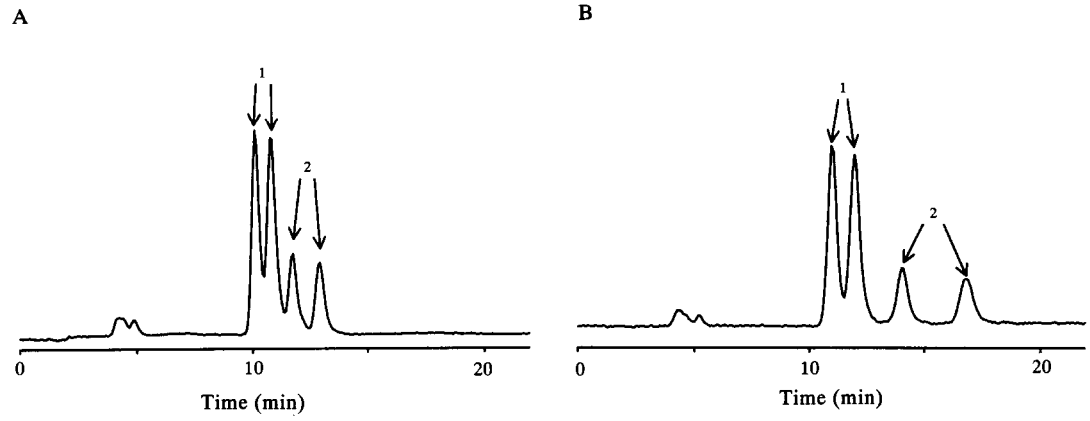


**Figure 4**
The effect of temperature on the enantioselective resolutions of OH-ME-MEX-OL and *p*-OH-MEX-OL. (A) Chromatography at ambient temperature; (B) chromatography at 0°C. 1 = The enantiomers of OH-ME-MEX-OL; 2 = *p*-OH-MEX-OL. See text for chromatographic conditions.

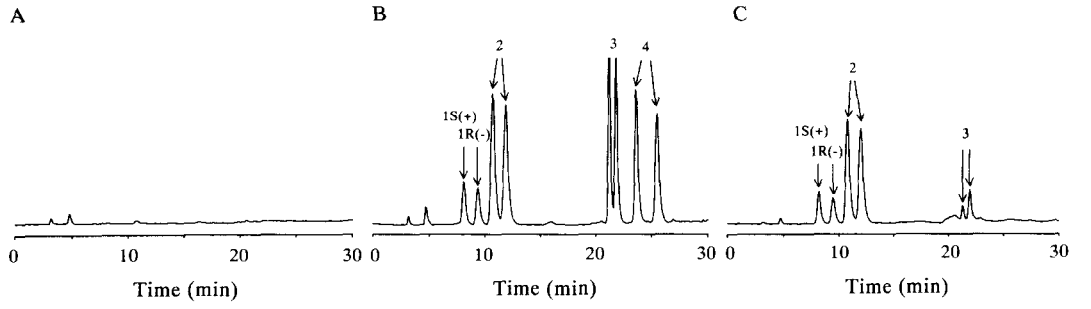


**Figure 5**
Determination of MEX, OH-ME-MEX, *p*-OH-MEX in (A) blank plasma; (B) plasma sample spiked with 0.5 μg $ml^{-1}$ each of MEX, OH-ME-MEX, *p*-OH-MEX; (C) plasma sample taken 2 h post-administration of an oral dose of 100 mg rac-MEX. 1S(+) = (*S*)-(+)-MEX; 1R(−) = (*R*)-(−)-MEX; 2 = I.S.; 3 = enantiomers of OH-ME-MEX; 4 = enantiomers of *p*-OH-MEX. See text for chromatographic conditions.

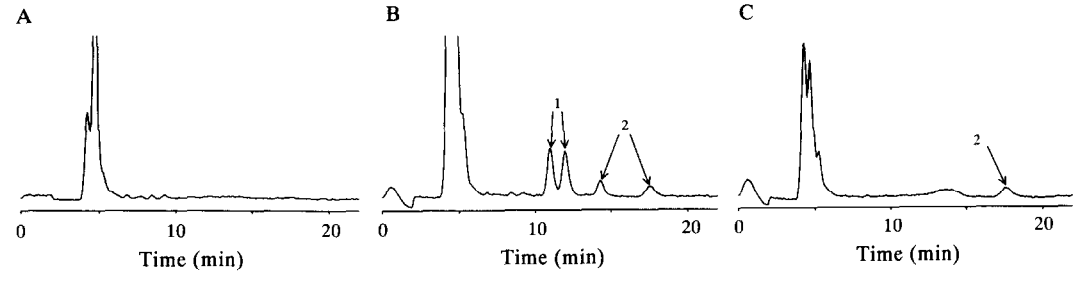


**Figure 6**
Determination of the enantiomers of OH-ME-MEX-OL and *p*-OH-MEX-OL in: (A) blank plasma; (B) plasma sample spiked with 0.2 μg $ml^{-1}$ each of OH-ME-MEX-OL and *p*-OH-MEX-OL; (C) plasma sample taken 2 h post-administration of an oral dose of 100 mg rac-MEX. 1 = The enantiomers of *p*-OH-MEX-OL; 2 = OH-ME-MEX-OL.

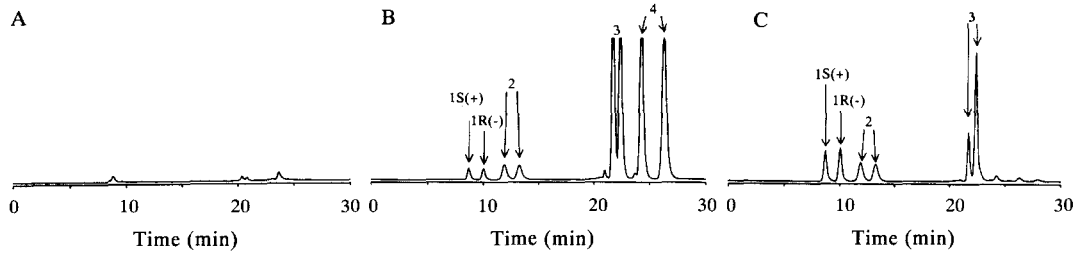


**Figure 7**
Determination of MEX, OH-ME-MEX, *p*-OH-MEX in (A) blank urine; (B) urine sample spiked with 0.5 μg $ml^{-1}$ each of MEX, OH-ME-MEX, *p*-OH-MEX; (C) 24-h urine sample from a patient receiving 100 mg rac-MEX twice a day. 1S(+) = (*S*)-(+)-MEX; 1R(−) = (*R*)-(−)-MEX; 2 = I.S.; 3 = enantiomers of OH-ME-MEX; 4 = enantiomers of *p*-OH-MEX. See text for chromatographic conditions.

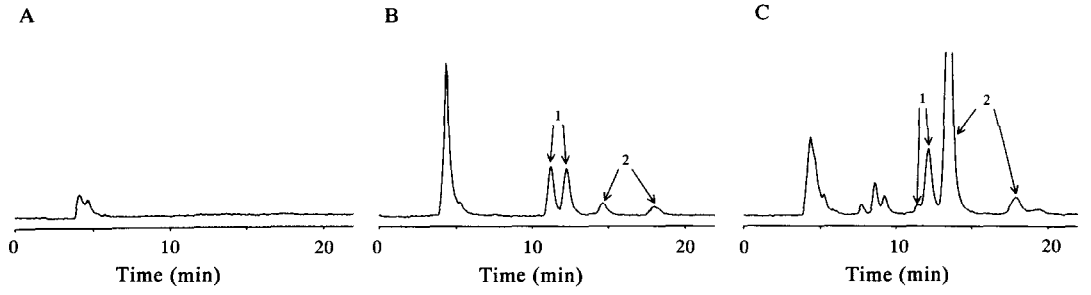


**Figure 8**
Determination of the enantiomers of OH-ME-MEX-OL and *p*-OH-MEX-OL in (A) blank urine; (B) urine sample spiked with 0.5 μg $ml^{-1}$ each of OH-ME-MEX-OL and *p*-OH-MEX-OL; (C) 24-h urine sample from a patient receiving 100 mg rac-MEX twice a day. 1 = The enantiomers of *p*-OH-MEX-OL; 2 = OH-ME-MEX-OL. See text for chromatographic conditions.

relief from cancer-related neuropathic pain are presented in Figs 5–8. Figures 5 and 6 present the chromatograms from plasma samples obtained for the analysis of MEX, OH-ME-MEX and *p*-OH-MEX (Fig. 5) and *p*-OH-MEX-OL and OH-ME-MEX-OL (Fig. 6). Figures 7 and 8 present the chromatograms from urine samples obtained for the analysis of MEX, OH-ME-MEX and *p*-OH-MEX (Fig. 7) and *p*-OH-MEX-OL and OH-ME-MEX-OL (Fig. 8).

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