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RESOLUTION OF ENANTIOMERS OF THE ANTIARRHYTHMIC DRUG ENCAINIDE AND ITS MAJOR METABOLITES BY CHIRAL DERIVATIZATION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Commercially available chiral columns were unable to provide adequate resolution of enantiomers of the antiarrhythmic drug encainide or its major metabolites. The homochiral derivatizing agent, (−)-menthyl chloroformate, was found to react at the tertiary piperidine nitrogen of racemic encainide providing two menthyl carbamate diastereomers. The individual diastereomers could be separated with baseline resolution on normal-phase high-performance liquid chromatography on a silica column. Structures of the derivatives were confirmed by electron impact mass spectrometry and ^{1}H NMR spectroscopy. The method was adapted for the chiral analysis of the major metabolites of encainide. The limit of sensitivity for racemic encainide was 10 ng on column and it was possible to detect a mixture containing (+)- and (−)-encainide in a ratio of 1:99. Preliminary studies indicated that (−)-encainide was O-demethylated to a greater extent than the (+)-enantiomer by rat liver microsomes.

INTRODUCTION

Encainide {**1**, Fig. 1; 4-methoxy-2′-[2-(1-methyl-2-piperidyl)ethyl]-benzanilide} is an antiarrhythmic drug that is extremely effective for suppres-

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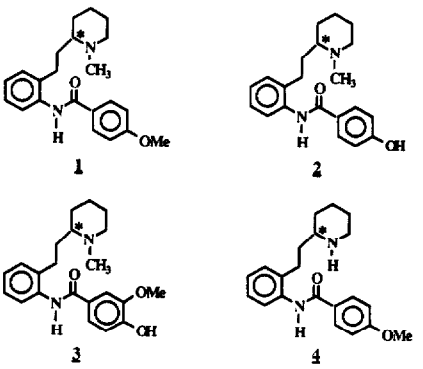

Fig. 1. Structures of (±)-encainide (**1**) and its metabolites (±)-ODE (**2**), (±)-MODE (**3**) and (±)-NDE (**4**). The asterisk indicates the position of the chiral center in the molecule.

sion of ventricular ectopic activity [1–4]. It is particularly useful for treating patients with recurrent life threating ventricular tachycardia [5]. Metabolism of encainide in human subjects occurs under genetic control of the debrisoquine type [6–9]. Extensive metabolizers convert encainide to O-demethylencainide (ODE, **2**; Fig. 1) and 3-methoxy-O-demethylencainide (MODE, **3**; Fig. 1) [6,9]. In poor metabolizers small amounts of ODE and N-demethylencainide (NDE, **4**; Fig. 1) have been detected [7]. However, no MODE is formed in these subjects [7]. ODE and MODE are potent antiarrhythmic agents in their own right [10–12] and NDE has weak antiarrhythmic activity [13]. It has been suggested that ODE and MODE contribute to the efficacy of encainide during long-term therapy in the extensive metabolizer phenotype [14].

Encainide possesses a single chiral center adjacent to the piperidine nitrogen and like many cardiovascular drugs it is normally administered to human subjects as a racemate. Little is known about possible enantioselectivity in the disposition of encainide. This is largely a consequence of the problems involved in resolution of the individual enantiomers of encainide and their metabolites. Prior to this study there were no chromatographic procedures available for the separation of enantiomers of encainide or its major metabolites. The resolution of drug enantiomers is normally carried out most efficiently by high-performance liquid chromatography (HPLC) [15,16]. Most of the methods that have been described involve an interaction between a heterochiral substrate and a homochiral ligand. This results in the formation of either dissociable complexes or covalent diastereomers. Dissociable complexes of individual enantiomers can be separated either by the use of chiral mobile phase additives or by the use of chiral bonded stationary phases [15–19]. Covalent diastereomers can normally be resolved by chromatography on achiral stationary phases. Numerous derivatizing reagents have been reported for the preparation of covalent diastereomers from individual enantiomers containing diverse functional

groups [15,16,20]. Unfortunately, encainide enantiomers could not be separated by chromatography on chiral stationary phases. Further, the molecule lacked a free functional group that would permit ready formation of chiral derivatives. We report a procedure for derivatization of (±)-encainide with a chiral chloroformate reagent. The resulting diastereomers can be readily separated on normal-phase HPLC. The procedure has been applied for the chiral analysis of (±)-encainide and its metabolites ODE, NDE and MODE. It has also been used to examine the enantioselective metabolism of (±)-encainide in rat liver microsomes.

EXPERIMENTAL

Chemicals

Racemic encainide, (+)-and (−)-enantiomers of encainide, MODE, ODE and NDE were supplied by Bristol-Myers (Wallingford, CT, U.S.A.). (−)-Menthyl chloroformate [(−)-MCF], N,N′-diisopropylethylamine and deuterated chloroform (C^2HCl_3, 99.96 atom-% ^2H) were obtained from Aldrich (Milwaukee, WI, U.S.A.). Diazomethane was generated just prior to use from N-nitrosomethylurea obtained from ICN (Plainview, NY, U.S.A.). HPLC-grade solvents were purchased from American Scientific Products (Atlanta, GA, U.S.A). All other chemicals were of reagent grade and were used without purification. Silica gel 60 Å was purchased from Fisher Scientific (Stone Mountain, GA, U.S.A.).

High-performance liquid chromatography

HPLC was carried out on two systems. System A consisted of a Waters (Milford, MA, U.S.A.) Model 510 gradient delivery system and a Valco injection valve fitted with 500-μl loop. The derivatives were monitored with a Lambda-Max Model 481 spectrophotometer at 261 nm. Chromatograms were recorded on a Shimadzu C-R3A recorder at a chart speed of 5 mm/min. Separations were carried out on an Ultrasphere (5 μm, 250 mm×4.6 mm; Altex) silica column at ambient temperature. The mobile phase was ethyl acetate–hexane–triethylamine (15:85:1, v/v). The flow-rate was maintained at 1 ml/min. System B consisted of a DuPont 870 pump module and 8800 gradient controller with a Hitachi 655A-40 autosampler and D2000 chromato-integrator. UV detection was carried out on a Waters Model 481 spectrophotometer. Chromatography was carried out on a semi-preparative Ultrasphere silica column (5 μm, 250 mm×10 mm; Altex) using a mobile phase that consisted of methanol –0.01 *M* aqueous ammonium acetate pH 5 (60:40, v/v). The flow-rate was maintained at 2 ml/min.

Mass spectrometry

Mass spectra were obtained on a VG 70/250 double focusing mass spectrometer. Fast atom bombardment (FAB) mass spectra were obtained with a xenon saddle-field gunning operating at 8 keV and glycerol was used as the matrix. Electron impact (EI) mass spectra were obtained at 70 eV.

NMR spectroscopy

The ^1H NMR spectra were obtained on a Bruker NR-300 Fourier transform NMR spectrometer operating at 300 MHz. NMR spectra were obtained in C^2HCl_3. Chemical shifts are reported in ppm relative to tetramethylsilane as an internal reference.

Derivatization of encainide and NDE

(±)-Encainide or (±)-NDE (5 mg) in acetonitrile containing N,N′-diisopropylethylamine (1 ml, 90:10, v/v) was heated at 60°C with 500 µl (−)-MCF solution in acetonitrile (10:90, v/v). After heating for 2 h, the acetonitrile was evaporated and the carbamate derivatives were purified on a small column of silica gel using 15% acetone in hexane. The EI mass spectrum of the menthyl carbamate derivative of racemic encainide (Fig. 2) showed ions at *m/z* 520 (M^+), *m/z* 337 ($M^{+\cdot}$−OCO−menthyl), 135 (amide cleavage), 84 (cleavage α-to the piperidine nitrogen). The ^1H NMR spectrum showed δ_H 7.83 (2 H, d, aromatic H-2, H-6), 7.3–7.10 (4 H, m, aromatic), 6.94 (2 H, d, aromatic H-3, H-5), 4.48 (1 H, m, menthyl CHO), 4.26 (1H, bm), 3.85 (3 H, s, OCH_3), 2.80–1.10 (22 H, m, aliphatic CH), 0.80–1.00 (9 H, m, $C(CH_3)_2$ and CH_3). The EI mass spectrum of racemic NDE carbamate showed a molecular ion at *m/z* 520.

Derivatization of (±)-encainide and (±)-NDE for analytical determina-

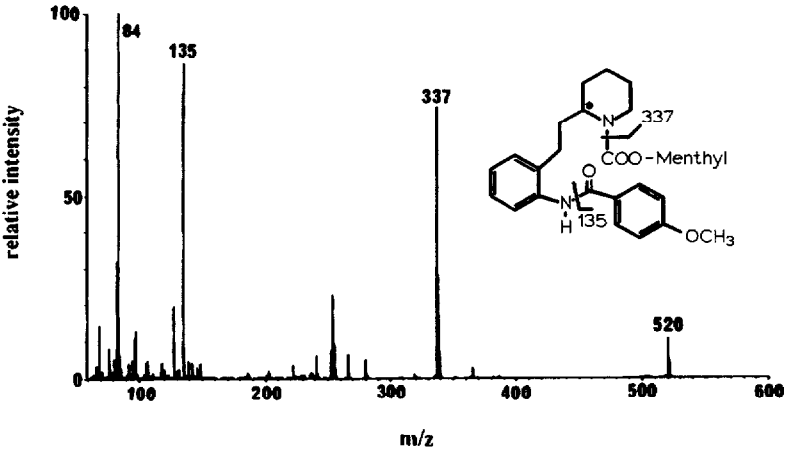

Fig. 2. EI mass spectrum of the menthyl carbamate derivative of racemic encainide.

tions was carried out as follows. The sample containing (±)-encainide or (±)-NDE (10 ng to 5 μg) was dissolved in acetonitrile containing N,N′-diisopropylethylamine (50 μl, 90:10, v/v) and to this was added (−)-MCF in acetonitrile (50 μl, 10:90, v/v). The solution was heated for 2 h at 60°C and the acetonitrile evaporated. The residue was dissolved in the initial mobile phase ready for HPLC analysis.

Derivatization of ODE and MODE

(±)-ODE and (±)-MODE (1 mg) were derivatized as described above for (±)-encainide. FAB mass spectra of the carbamate derivatives showed protonated molecular ions at *m/z* 689 and 719 respectively (data not shown). This suggested that bis-derivatization had occurred by reaction on both the piperidyl nitrogen and on the free phenol.

(±)-ODE and (±)-MODE (1 mg) were dissolved in methanol (200 μl) and freshly prepared etheral diazomethane (2 ml) was added. After standing for 2 h at 4°C the solvent was evaporated. Methylation was shown to be complete by HPLC analysis on system B. Methylated (±)-ODE and (±)-MODE showed molecular ions in their EI mass spectra at *m/z* 352 and *m/z* 382 respectively. The methyl ethers were converted to carbamate derivatives as described above for (±)-encainide. The EI mass spectra for the methylated carbamate derivatives of (±)-ODE and (±)-MODE showed molecular ions at *m/z* 520 and *m/z* 550, respectively.

Derivatization of (±)-ODE and (±)-MODE for analytical determinations was carried out as follows. The sample containing (±)-ODE or (±)-MODE (10 ng to 5 μg) was dissolved in methanol (50 μl) and ethereal diazomethane (200 μl) added. After standing for 2 h at 4°C the solvent was evaporated. Derivatization with (−)-MCF was carried out as described above for (±)-encainide and (±)-NDE.

Preparation of rat liver microsomes

Two male rats (Sprague–Dawley, Harlan) were fasted overnight and sacrificed by decapitation. The portal vein was flushed with 0.15 *M* KCl (ca. 50–100 ml) to clear the blood. The liver was removed and microsomes prepared using essentially the method of Remmer et al. [21].

Incubation of encainide with rat liver microsomes

The incubation mixture (10 ml total volume) contained 0.05 *M* Tris–HCl (pH 7.4), 0.01 *M* $MgCl_2$, 0.15 *M* KCl, 2 mg/ml isocitric acid, 4 μl/ml isocitric acid dehydrogenase, 25 μ*M* (±)-encainide and rat liver microsomes at a protein concentration of 1 mg/ml. The reaction was initiated by the addition of 1 mg/ml of NADPH and incubated at 37°C. After 90 min, the reaction was stopped by the addition of ethyl acetate. The microsomal incubate was evaporated to dryness and dissolved in 10 ml of water. The solution was applied to

a C_{18} Sep-Pak pre-washed with 5 ml of methanol followed by 5 ml of water. The mixture of metabolites was eluted from the Sep-Paks with 5 ml of methanol. The methanol solution was evaporated to dryness, the metabolites were redissolved in methanol–water (50:50, v/v) and purified by HPLC using system B. The purified metabolites and residual encainide were derivatized with (−)-MCF as described above and analyzed on HPLC system A.

RESULTS

Resolution of encainide enantiomers

The UV chromatogram obtained for the carbamate derivatives prepared from racemic encainide on HPLC system A is shown in Fig. 3a. Baseline separation was obtained for the two diastereomers with a separation coefficient (α) of 1.07 and resolution factor (R) of 1.41. In further experiments it was shown that the ratio of the peak area of (+)-encainide carbamate to (−)-encainide carbamate was 1.002 ± 0.008 ($n=5$). Thus, there was no kinetic resolution of the two enantiomers under the reaction conditions. The pure (+)- and (−)-enantiomers of encainide were separately converted to their carbamate derivatives with (−)-MCF and also analyzed on HPLC system A. The diastereomer from (+)-encainide (Fig. 3b) eluted before that from the (−)-enantiomer (Fig. 3c). Racemic encainide could be resolved with a limit of sensitivity of 10 ng on column (signal-to-noise ratio 10:1) when the UV was monitored at 261 nm. It was possible to readily detect 1% of the (+)-enantiomer in the presence of 99% of the (−)-enantiomer.

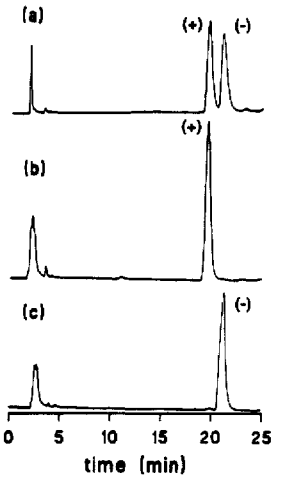

Fig. 3. Chromatograms obtained on HPLC system A for carbamates obtained from (a) racemic encainide, (b) (+)-encainide and (c) (−)-encainide.

Resolution of enantiomers of ODE, MODE and NDE

The diastereomers of the bis-carbamate derivatives of ODE and MODE could not be resolved by chromatography on HPLC system A. Bis-derivatization was prevented by blocking the free phenolic groups as methyl ethers by reaction of (±)-ODE and (±)-MODE with diazomethane. The carbamate derivatives of the methylated products of (±)-ODE and (±)-MODE could be readily resolved by chromatography on HPLC system A (Fig. 4a and b). Racemic NDE

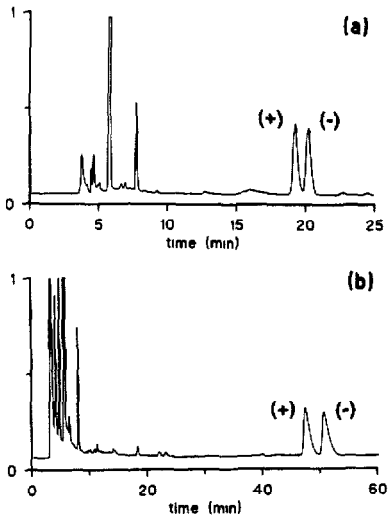

Fig. 4. Chromatograms obtained on HPLC system A for the carbamate diastereomers obtained from (a) methylated (±)-ODE and (b) methylated (±)-MODE.

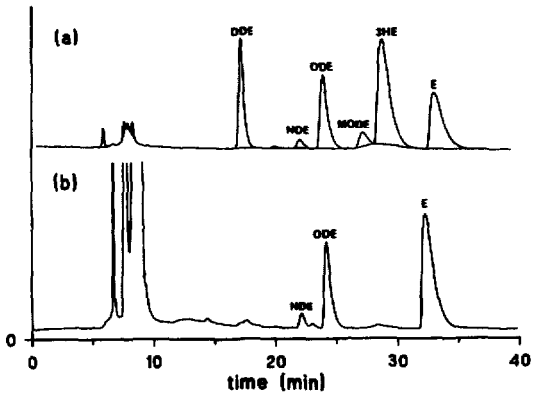

Fig. 5. Chromatograms obtained on HPLC system B for (a) synthetic encainide derivatives N,O-di-demethylencainide (DDE), NDE, ODE, MODE, 3-hydroxyencainide (3HE) and encainide (E). (b) Mixture of metabolites isolated from rat liver microsomal incubates.

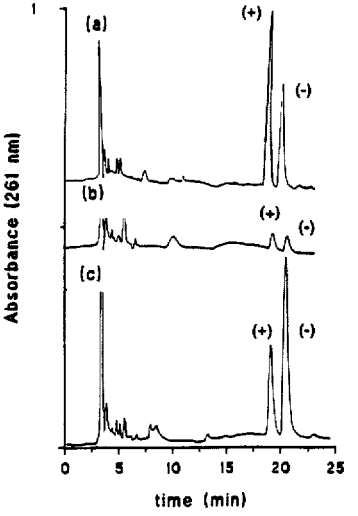

Fig. 6. Chromatograms obtained in HPLC system A for carbamate diastereomers of encainide and metabolites isolated from rat liver microsomal incubates. (a) encainide (b) NDE and (c) ODE.

formed carbamate diastereomers that could be resolved on HPLC system A (data not shown).

Analysis of encainide and metabolites from microsomal incubates

The mixture of metabolites isolated from rat liver microsomal incubates were analyzed on HPLC system B (Fig. 5b). A chromatogram obtained from a mixture of six synthetic encainide analogues run under identical conditions is shown in Fig. 5a. It was evident from a comparison of the two chromatograms that ODE was the major metabolite. ODE, NDE and residual encainide from the incubation mixture were isolated using HPLC system B. The isolated ODE was methylated with diazomethane and converted to the menthyl carbamate derivative. NDE and encainide were converted to carbamate diastereomers directly. The carbamate derivatives were then analyzed by HPLC on system A. This revealed that the residual encainide had an excess of (+)-enantiomer (Fig. 6a), NDE was present as a racemic mixture (Fig. 6b) and ODE had an excess of (−)-enantiomer (Fig. 6c).

DISCUSSION

Commercially available chiral bonded stationary phase columns including β-cyclodextran, Pirkle (phenylglycine), chiralgel (OB, OD, OC) and α_1-acid glycoprotein were unsuccessful in the separation of encainide enantiomers. Preparation of the diastereomers was difficult as the only functional group available for chiral derivatization was a tertiary amine. Chiral chloroformates

have been used previously for the conversion of chiral drugs to their respective diastereomers so that they can be readily resolved by HPLC [20,22–24]. There are a number of reports on the use of (−)-MCF for the derivatization of secondary alcohols [25–27] and secondary amines [24, 27–29]. However, there are no reports on the use of chiral chloroformates for derivatization of tertiary amines. Reactions between tertiary amines and chloroformates have generally been used as the first step in a two-step procedure that results in N-dealkylation. The first step results in the formation of a carbamate derivative [30,31] that can subsequently be hydrolyzed in a second step to give a secondary amine [31]. Encainide could react with a chloroformate in two distinct ways; first, by cleavage of the piperidine ring to give open-chain compounds and second, by loss of the N-methyl group to give the corresponding carbamate. We reasoned that if this latter route of reaction predominated and if a chiral chloroformate reagent was used, this would provide an efficient method for the preparation of carbamate diastereomers of encainide.

Reaction of (±)-encainide with the chiral reagent (−)-MCF proceeded almost exclusively by the desired N-dealkylation pathway (Fig. 7) and led to the formation of carbamate derivatives in >95% yield (as judged by HPLC). No enantioselectivity was observed in the reaction. Separation of the enantiomers of the O-demethylated metabolites of encainide proved to be more difficult. When treated with (−)-MCF both (±)-ODE and (±)-MODE gave bis-derivatives that could not be separated using a variety of solvent systems. It appeared that the increased hydrophobicity together with the complex stereochemistry of the bis-derivative interfered in the chromatographic resolution of the enantiomers on the HPLC systems that were employed. It was evident that bis-derivatization could be prevented by methylating the free phenolic group in the ODE and MODE. It was recognized that this would convert ODE to encainide. However, this was not considered to be a problem as it was

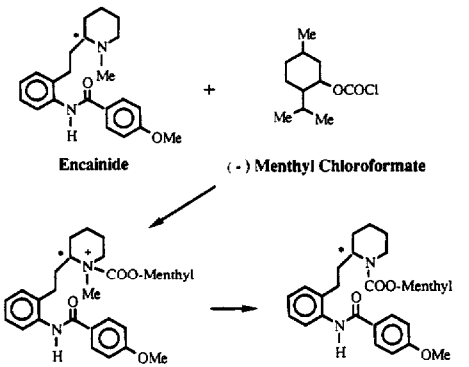

Fig. 7. Scheme for preparation of carbamate derivatives by reaction of (±)-encainide with (−)-MCF.

anticipated that the metabolites would be separated from residual encainide prior to derivatization. Attempts to methylate (±)-ODE and (±)-MODE using dimethyl sulfate or methyl iodide resulted in the formation of very polar compounds. The ^{1}H NMR spectra and FAB mass spectra indicated quaternary ammonium salts had been formed during the reaction. Subsequently, it was found that methylation could be accomplished in high yield under very mild conditions using ethereal diazomethane as the reagent. The methyl ether prepared from (±)-ODE was identical in all respects with (±)-encainide, so diastereomeric carbamates prepared by reaction with (−)-MCF could be separated by chromatography on HPLC (Fig. 4a). The diastereomeric carbamates prepared by reaction of the methyl ether from (±)-MODE with (−)-MCF could also be readily separated (Fig. 4b).

The methodology developed for the separation of encainide enantiomers has been applied to a study of (±)-encainide metabolism in a microsomal system. Examination of the incubation mixture by chromatography on HPLC (Fig. 5) revealed that ODE and NDE were the major products together with residual starting material. HPLC separation of the carbamate diastereomers obtained from unchanged encainide revealed the presence of excess (+)-enantiomer (Fig. 6a). The carbamates derived from NDE showed no enantioselectivity (Fig. 6b) but those derived from ODE had an excess of (−)-enantiomer (Fig. 6c). Thus, (−)-encainide underwent O-demethylation to a greater extent than (+)-encainide in the microsomal incubation, so that (−)-ODE was formed preferentially to (+)-ODE. There was no enantioselectivity in N-demethylation of (±)-encainide to NDE. These results are somewhat surprising. It may have been expected that if enantioselective metabolism had occurred, it would have taken place proximal to the site of chirality. The observed enantioselectivity in demethylation of the aromatic methyl ether occurred at a site that was quite remote from the center of chirality. A detailed investigation of this observation is currently under way and will be reported separately.

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