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Note

Stereoselective analysis of the enantiomers of ethotoin in human serum using chiral stationary phase liquid chromatography and gas chromatography–mass spectrometry

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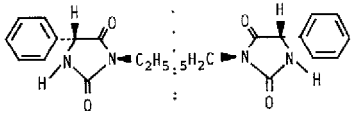
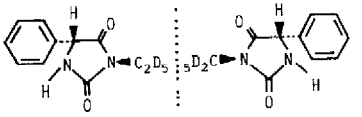
Ethotoin (ETT, Fig. 1) [1, 2], 3-ethyl-5-phenylhydantoin, has been reported to have a lower anticonvulsant activity in epileptic patients than phenytoin (PHT). Although ETT lacks the typical side-effects of PHT, such as gingival hyperplasia, hirsutism and ataxia, it is not considered to be a primary antiepileptic agent. Recently, Carter et al. [3] reported that seizure was controlled, without side-effects, by ETT administration in sixteen out of seventeen patients who suffered from a variety of seizure disorders. Little information on the disposition of ETT in patients has been reported because of the lack of an adequate quanti-



Ethotoin



[D_5]-Ethotoin

Fig. 1. Chemical structure of ethotoin.

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fication method of ETT for the patients taking various other antiepileptic agents [4–6]. Although Yonekawa et al. [7] reported a gas chromatographic (GC) method for the determination of ETT from human plasma, their method could not be used for the analysis of the barbiturates. We have previously developed a rapid simultaneous quantitation method for ETT, carbamazepine (CBZ), phenobarbital (PB), PHT and primidone (PMD) in serum using a selected-ion monitoring (SIM) technique [8].

ETT has a chiral centre and is used therapeutically as the racemic mixture. It has been reported that the enantiomers of many agents exhibit different pharmacokinetic and/or pharmacodynamic characteristics [9, 10]. Although many attempts to convert enantiomers into diastereomers in pharmacokinetic studies have been reported [11, 12], the use of the chiral stationary phases for high-performance liquid chromatography (HPLC) has been studied only infrequently [13, 14]. In this report, we describe a method of direct determination of ETT enantiomers in serum using chiral stationary phase HPLC and gas chromatography–mass spectrometry (GC–MS).

EXPERIMENTAL

Materials

Racemic [$^2H_5$]ETT was kindly supplied by Dainippon Pharmaceutical (Osaka, Japan). Racemic ETT of a commercially available grade was purchased from Dainippon Pharmaceutical. MethElute, a methylating agent for on-column derivatization of drugs in GC, was purchased from Pierce (Rockford, IL, U.S.A.). MethElute was labelled to contain a 0.2 *M* solution of trimethylanilinium hydroxide in anhydrous methanol. Organic solvents were distilled before use. Other chemicals were of reagent grade and purchased from Wako (Osaka, Japan).

Extraction procedure

To a 100-μl portion of serum in a 10-ml glass test-tube, 1 ml of a saturated solution of sodium chloride and 1 ml of chloroform containing 4 μg of racemic [$^2H_5$]ETT as an internal standard were added. The sample was mixed for 10 s by a vibration mixer. After brief centrifugation, the upper aqueous layer was aspirated off. The remaining chloroform layer was then transferred to a 2-ml glass sample tube and evaporated to dryness under vacuum at 50°C. The residue was redissolved in 50 μl of ethanol, and a 25-μl aliquot of the resultant solution was injected into the HPLC system to separate optically active ETT. The column effluents of each enantiomer were collected in a 10-ml test-tube on the basis of the retention times of the racemic ETT standard sample, which was injected before collection was started. The effluents from the overlapping region were not collected on the basis of the retention times. Each enantiomer collected was checked for its optical purity by HPLC. Each effluent was evaporated to dryness under vacuum at 50°C. The residue was then dissolved in 25 μl of MethElute, and a 1-μl aliquot of the resultant solution was injected into the GC–MS system.

HPLC conditions

An LC-3A HPLC system (Shimadzu, Kyoto, Japan), equipped with a UV detector set at 254 nm and a C-R1A data terminal (Shimadzu), were employed. The chiral stationary phase column was a 250 mm×4.6 mm I.D. Chiralcel CA-1 column consisting of cellulose triacetate (Daisel Chemical, Himeji, Japan). The mobile phase was 95% ethanol and was delivered isocratically at 0.5 ml/min at the column temperature of 35 °C.

GC–MS conditions [8]

A QP-1000 GC–MS system (Shimadzu) was used. The GC part was equipped with a 1.2 m×3 mm I.D. glass column of 3% OV-17 on Gas Chrom Q. The flow-rate of helium was 50 ml/min. The temperature of the injection port, the transfer line to the mass spectrometer and the ion source of the mass spectrometer were 300, 250 and 250 °C, respectively. The following programme for the column temperature was employed; the initial temperature was 215 °C with a hold time of 1.5 min; the temperature was then raised at 10 °C/min to a final temperature of 250 °C.

Mass spectra were obtained in an electron-impact mode at an electron energy of 70 eV. SIM was performed at m/z 218 and 223 for ETT and [$^2H_5$]ETT, respectively. Peak-area ratios of ETT to [$^2H_5$]ETT were calculated to obtain the standard curves.

Standard curves

Known amounts of racemic ETT up to 100 μg/ml were added to a blank serum. The concentrations of the enantiomers of ETT were estimated by the peak-area ratios of each enantiomer of ETT to the respective enantiomer of [$^2H_5$]ETT. The standard curves were obtained for each set of the serum samples.

Clinical study

A female patient, 17 years old, participated in the study. Ethical aspects of the present study were guided by the Declaration of Helsinki, since an institutional review board has not been established in our institution yet. An informed consent was obtained from her guardian. The subject was given a 2000-mg powder of racemic ETT. Serum samples were collected at various times and stored at −20 °C until analysis.

Data analysis

The serum concentration–time profiles for the racemate and both enantiomers were fitted to a one-compartment model using a non-linear regression analysis technique in order to obtain the pharmacokinetic parameters.

RESULTS AND DISCUSSION

Resolution of enantiomers of ETT

A typical chromatogram illustrating the resolution of ETT enantiomers achieved on the Chiralcel CA-1 column is shown in Fig. 2A. The retention time of the *d*-enantiomers of ETT and [$^2H_5$]ETT was 11.9 min and that of the *l*-

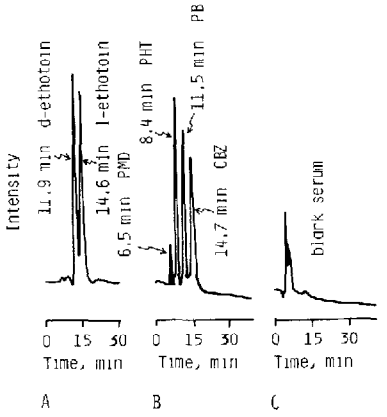

Fig. 2. Typical HPLC chromatograms of ethotoin (A), primidone, phenytoin, phenobarbital and carbamazepine (B) and blank serum (C).

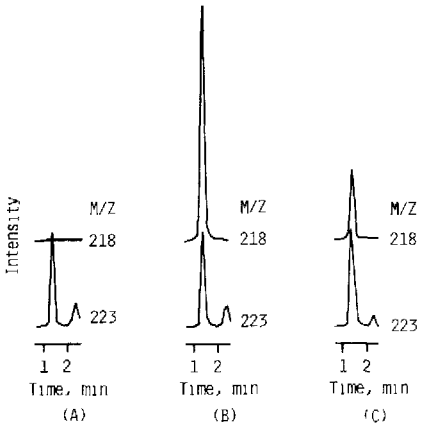

Fig. 3. Typical SIM chromatograms of a blank serum spiked with an internal standard (A), a blank serum spiked with 50 μg/ml *d*-ETT (B) and a patient sample containing 36.8 μg/ml *d*-ETT (C).

enantiomers was 14.6 min. These results show that [$^2H_5$]ETT is a good internal standard not only for extraction and GC–MS analysis, but for separation on the chiral stationary phase for HPLC. Other antiepileptic agents that are frequently prescribed with ETT were eluted at retention times of 6.5, 8.4, 11.5, and 14.7 min for primidone, phenytoin, phenobarbital, and carbamazepine, respectively (Fig. 2B). Thus, a combined procedure of HPLC and GC–MS is needed to quantitate the optically active ETT. The column effluents of each enantiomer were collected for GC–MS analysis on the basis of the retention times of the standard sample injected before collection was started. Although the retention times of the enantiomers were also checked after collection, no difference was observed between the retention times before and after collection.

Gas chromatography–mass spectrometry

The GC–MS assay described here is the same as that used in the racemic mixture of ETT assay previously reported [8]. Mass spectra of racemic ETT exhib-

ited a peak of m/z 218 in the electron-impact mode. Because the spectra of both enantiomers were the same as that of racemic ETT, SIM was performed at m/z 218 and 223 for the enantiomers of ETT and [$^2H_5$]ETT, respectively.

Representative SIM profiles of the HPLC effluent of the human serum are shown in Fig. 3. The background peaks of blank human serum were few and completely separated from the peaks of ETT and [$^2H_5$]ETT.

Validation of the method

The recovery of racemic ETT through the whole procedure without HPLC resolution of the enantiomers was previously reported to be 95.8% at an ETT concentration of 50 μg/ml [8]. No effort was made to increase the recovery in this study, and 8 ng of ETT, corresponding to ca. 2 μg/ml, could be measured quantitatively [8]. Linear relationships were obtained up to a serum concentration of 50 μg/ml for the enantiomers of ETT, with correlation coefficients of 0.9998. Inter-day coefficients of variation for quantitation of racemic ETT were found in the previous study [8] to be 0.47–3.60% at serum concentrations of 10–100 μg/ml.

TABLE I

PHARMACOKINETIC PARAMETERS OF ETHOTOIN

Abbreviations: k_a = absorption rate constant; k_{el} = elimination rate constant; V_d = volume of distribution, F = fraction absorbed; $AUC_{0-\infty}$ = area under the curve from time zero to infinity; MRT = mean residence time.

	k_a (h^{-1})	k_{el} (h^{-1})	V_d/F (l)	$AUC_{0-\infty}$ (μg/ml/h)	MRT (h)
Racemate	1.84	0.034	41 7	1490	32.7
d-Enantiomer	2.50	0.042	43.7	740	49.7
l-Enantiomer	2.12	0.054	55 6	500	50.5

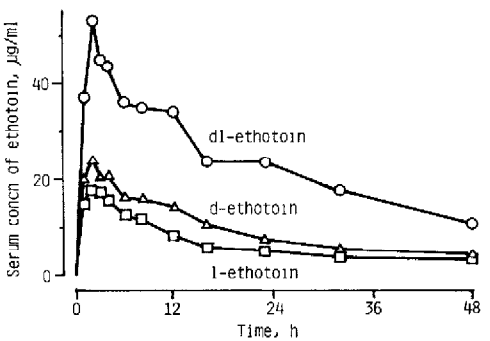

Fig 4 Serum racemic ETT (○), *d*-ETT (△), and *l*-ETT (□) concentration profiles of a patient who had received an oral dose of 2000 mg of racemic ethotoin.

Pharmacokinetic study

Fig. 4 shows the serum concentrations of racemic ETT and the enantiomers of ETT after oral administration of 2000 mg of racemic ETT to one patient. Pharmacokinetic parameters, estimated by the non-linear regression analysis and listed in Table I, indicate that the area under the time–concentration curve of the *d*-enantiomer of ETT was greater than that of the *l*-enantiomer, although no statistical evaluation could be made because the data were based on only one patient.

CONCLUSION

The method decribed has the advantage of high specificity. The method employing the chiral column for HPLC, which is not as expensive as the non-chiral column now used, eliminates the complicated and time-consuming procedures needed to produce the diastereomers of the drug.

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