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SIMULTANEOUS DETERMINATION OF THE ENANTIOMERS OF TOCAINIDE IN BLOOD PLASMA USING GAS—LIQUID CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

Tocainide is a new experimental antiarrhythmic agent used clinically as the racemic mixture of two enantiomers. Since the optical isomers may differ in their efficacy and toxicity, we have initiated studies on the stereoselective disposition of tocainide. For this purpose, an assay was developed for the simultaneous determination of the enantiomers of tocainide in blood plasma. Alkalinized 1-ml plasma samples containing tocainide and an internal standard, 2-amino-2′,6′-acetoxylidide, are extracted with ethyl acetate. The organic extract is treated with the chiral reagent (*S*)-α-methoxy-α-trifluoromethylphenylacetyl chloride, and the resulting derivatives are resolved and quantified by gas—liquid chromatography with electron-capture detection. Calibration data were fitted by least-squares power curves of the form: drug enantiomer/internal standard peak area ratio = $A \times C^B$ where A and B were constants and C was the concentration of tocainide enantiomer. The lower limit of sensitivity of the assay was 10 ng/ml of each enantiomer. Intra-assay coefficients of variation were 3.3 and 2.1% for (*R*)-(—)-tocainide at concentrations of 0.125 and 1.25 μg/ml, respectively, and 3.4 and 2.4% for the (*S*)-(+) enantiomer at the same concentrations. Diazepam may interfere with the determination of (*R*)-(—)-tocainide if concentrations smaller than 1 μg/ml of this enantiomer are measured in the presence of higher-than-therapeutic (> 1.5 μg/ml) concentrations of diazepam.

INTRODUCTION

Tocainide (2-amino-2′,6′-propionoxylidide, TOC, Fig. 1), a primary-amine analogue of lidocaine, is a new antiarrhythmic agent often useful in the treatment of life-threatening arrhythmias unresponsive to conventional therapy [1]. Unlike lidocaine, however, TOC is effective after oral administration. This difference between the two drugs is the result of structural modification of

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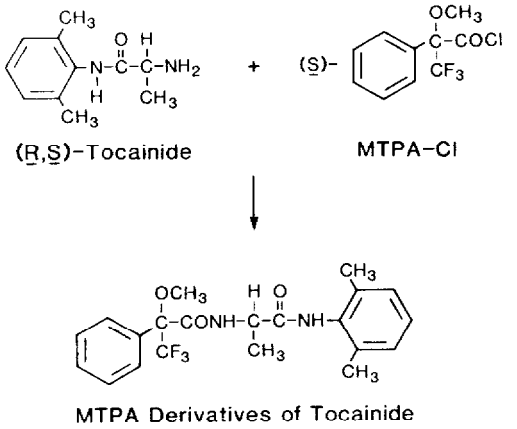


Fig. 1. Reaction of tocainide with (*S*)-MTPA-Cl.

TOC which makes it less susceptible to hepatic metabolism. The chemical structure of TOC (Fig. 1) includes an asymmetric center, and the drug is administered clinically as the racemic mixture. Studies in animals [2] have demonstrated that the enantiomers may differ in their antiarrhythmic efficacy and toxicity. This finding prompted us to undertake studies on the stereoselective disposition of tocainide [3, 4]. Planned pharmacokinetic studies require that we measure each enantiomer in serum, in the presence of the other, at concentrations as low as 50 ng/ml. In this communication, we describe the method developed for this purpose.

EXPERIMENTAL

Chemicals

Racemic and (*R*)-(−)-TOC hydrochloride were provided by Astra Pharmaceutical Products (Framingham, MA, U.S.A.). (*S*)-(+)-TOC hydrochloride was synthesized via a published procedure [2]. 2-Chloro-2′,6′-acetoxylidide and (*S*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetic acid ((*S*)-MTPA) were purchased from Aldrich (Milwaukee, WI, U.S.A.). (*S*)-MTPA was converted to the corresponding acid chloride, (*S*)-MPTPA-Cl, as described previously [5]. 2-Amino-6′-chloro-*o*-acetotoluidide (ACAT) hydrochloride was purchased from the ABC Library of Rare Chemicals, Aldrich. Ethyl acetate, distilled-in-glass grade, was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All other reagents were of analytical grade quality.

2-Amino-2′,6′-acetoxylidide (GX) was synthesized as follows: 2-chloro-2′,6′-acetoxylidide (1.0 g) was added to concentrated ammonium hydroxide (10 ml), and the mixture was stirred at room temperature for 24 h. This solution was extracted with diethyl ether (20 ml, three times) and the combined ether extracts were extracted with 1 *M* hydrochloric acid (20 ml, twice). The acidic aqueous extracts were combined, cooled in ice, and saturated with sodium hydroxide. The resulting alkaline aqueous solution was extracted with dichloromethane (25 ml, three times), the combined organic extracts were washed with water (25 ml, twice) and dried (potassium carbonate). The solvent was

evaporated at reduced pressure, and the solid obtained was dissolved in 50 ml dry diethyl ether. Hydrogen chloride gas was bubbled through the solution until the precipitation of GX hydrochloride ceased. The precipitate was filtered and washed with diethyl ether. The yield was 53%. GX free base was prepared by extracting an alkalinized (pH 14) aqueous solution of the hydrochloride with dichloromethane. Evaporation of the organic solvent gave a colorless solid, m.p. 77—80° C (literature value [6] m.p. 78—80° C).

(*R*,*S*)-TOC free base was similarly obtained from the hydrochloride and had a m.p. of 55—56° C.

Working solutions

Human plasma samples containing racemic TOC at several concentrations were prepared by appropriate dilutions of an aqueous stock standard solution containing TOC hydrochloride at 1.0 mg/ml free base concentration. GX hydrochloride was dissolved in distilled water to give a free base concentration of 16.6 μg/ml. (*S*)-MTPA-Cl was dissolved in ethyl acetate to give a concentration of 1 mg/ml.

Chromatography

A Hewlett-Packard (Avondale, PA, U.S.A.) Model 5736A gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. The stationary phase was 3% SP 2250-DA on 100-120 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.) contained in a 2 m × 2 mm I.D. glass column. Oven temperature was 255° C, injector temperature 300° C, and detector temperature 350° C. The carrier gas was nitrogen at a flow-rate of 30 ml/min.

Assay procedure

The sample, 1.0 ml, was placed in a 12-ml conical glass centrifuge tube and 50 μl of the internal standard GX solution, 1.5 ml ethyl acetate, and ca. 200 mg of a 4:1 mixture of sodium chloride and sodium carbonate were added. The mixture was swirl-mixed (vortex) for 60 sec, followed by centrifugation at 500 *g* for 5 min. The upper organic layer was transferred to a 12-ml screw-cap glass centrifuge tube and 10 μl of pyridine and 50 μl of the (*S*)-MTPA-Cl solution were added. The tube was tightly capped, swirl-mixed for 5 sec, and placed in a water bath at 70° C for 30 min. The tube was then cooled in an ice bath for 5 min, and 1 ml of 1 *M* hydrochloric acid was added. The mixture was swirl-mixed for 30 sec, followed by centrifugation for 30 sec. The organic layer was transferred to a 5-ml centrifuge tube, and 1 ml of 15% sodium carbonate solution was added. The mixture was swirl-mixed for 30 sec, and centrifuged for 30 sec. The organic layer was transferred to a 5-ml conical glass tube and evaporated to dryness at 50—60° C under a stream of nitrogen. The residue was stored at —20° C, and reconstituted with 100 μl of ethyl acetate immediately before gas—liquid chromatographic (GLC) analysis. Injection volumes were 0.5—6 μl.

Stability of derivatives

A 1-ml sample of water containing 50 μg of TOC was added to 100 μl of an aqueous solution containing 166 μg of GX and the mixture was processed

according to the assay procedure described above. The residue obtained upon evaporation of the solvent was reconstituted in 2 ml of ethyl acetate. A series of fourteen 100-μl aliquots of the resulting solution were evaporated to dryness in separate tubes. Two samples were reconstituted with 100 μl of ethyl acetate and analyzed by GLC immediately, while the remaining dried samples were stored for 6, 24, or 48 h at 4°C or −20°C. Each set of storage conditions was studied in duplicate. Absolute peak areas and TOC/GX area ratios were determined.

Recovery studies

A 1-ml sample of plasma, 1.5 ml of a stock ethyl acetate solution containing TOC free base at 0.667 μg/ml and GX free base at 0.553 μg/ml, and ca. 200 mg of a 4:1 mixture of sodium chloride and sodium carbonate were added to a centrifuge tube. The mixture was swirl-mixed for 60 sec, followed by centrifugation for 5 min. A 750-μl aliquot of the organic layer was treated with (*S*)-MTPA-Cl as described under Assay procedure. The derivatized mixture was analyzed by GLC, and the peak areas obtained were compared to the peak areas obtained when 750-μl aliquots of the stock ethyl acetate solution were derivatized with (*S*)-MTPA-Cl without prior extraction.

Calibration curves and detector response

Standard curves were constructed by analyzing a series of plasma samples of known racemic TOC concentration in the range 20 ng/ml—5.0 μg/ml.

Detector response linearity was studied as follows: the (*S*)-MTPA derivatives of TOC and GX were separately prepared. The derivative of TOC was serially diluted with an ethyl acetate solution of the derivative of GX in such a manner that the resulting samples contained the same fixed concentration of the GX derivative, and the concentration of the TOC derivative varied in the range which would be obtained in the analysis of 52 ng/ml—5.0 μg/ml plasma TOC samples.

Precision

Within-day precision was determined by analyzing sets of ten replicate plasma samples containing racemic TOC at 250 ng/ml and at 2.5 μg/ml concentrations. Day-to-day variability was determined by analyzing replicate samples containing TOC at a concentration of 2.5 μg/ml on ten separate days.

Quantitative evaluation of assay data

The TOC isomer: GX peak area ratios were used to construct standard curves. Calibration data were fitted by least-squares power curves through the origin of the form: peak area ratio = $A \times C^B$, where A and B were constants and C was the concentration of the TOC enantiomer.

Interference studies

Aliquots of ethyl acetate solutions of diazepam and nordiazepam at a concentration of 1.0 μg/ml were injected into the gas—liquid chromatograph and the peak areas obtained were compared to peak areas obtained from injections of the same solutions after extraction with 1 *M* hydrochloric acid. Triplicate injections of each solution were performed.

RESULTS

Fig. 2 shows the chromatogram of the MTPA derivatives of TOC and GX, and results of the analysis of a patient sample. When plasma from subjects not receiving TOC was analyzed, no interfering peaks were observed. In preliminary studies OV 17 GLC columns were used, but it was found that better chromatography was achieved on SP-2250 DA stationary phase. Retention times of the MTPA derivatives were: (*R*)-(−)-TOC, 8.9 min; (*S*)-(+)-TOC, 10.2 min; GX, 12.6 min.

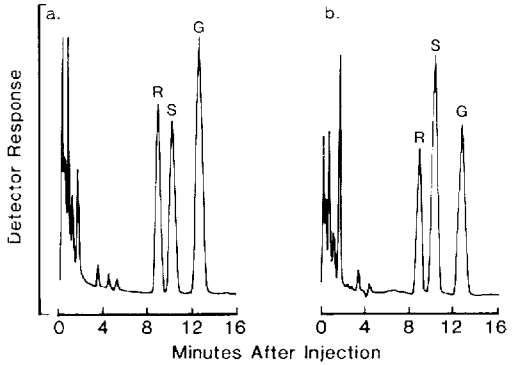


Fig. 2. Chromatograms of (a) the MTPA derivatives of standards of (*R*)-(−)-TOC (R), (*S*)-(+)-TOC (S), and GX (G); (b) extract of serum from patient receiving chronic TOC therapy assayed as described in Experimental. Concentration of (*R*)-(−)-TOC 1.54 μg/ml; (*S*)-(+)-TOC 3.73 μg/ml.

Recoveries of (*R*)-(−)- and (*S*)-(+)-TOC and of GX were 90.4 ± 6.8 (S.D.), 89.2 ± 6.7, and 89.6 ± 8.2, respectively (n = 5). In the derivatization procedure, the use of larger amounts of MTPA-Cl, e.g., 10—250 times that given in the assay procedure, was evaluated. No increase in the yield of derivatives was found, and interfering peaks appeared in the chromatograms.

TABLE I

STABILITY OF TOCAINIDE-MTPA DIASTEREOMERS AS A FUNCTION OF STORAGE TIME AND TEMPERATURE

Time (h)	Enantiomer response*			
	4° C		−20° C	
	(*R*)-(−)	(*S*)-(+)	(*R*)-(−)	(*S*)-(+)
0	797**	710**	797**	710**
6	813	724	812	725
24	813	727	823	735
48	821	736	823	736

*Ratio of tocainide enantiomer peak area to GX peak area × 1000. Tocainide enantiomer concentration was 1.25 μg/ml; mean of duplicate samples.
**Baseline samples were analyzed immediately after preparation.

TABLE II

TYPICAL CALIBRATION DATA

Least-squares power curves: tocainide (*R*)-(—) enantiomer: GX peak area ratio = 1704 (tocainide (*R*)-(—) enantiomer)$^{0.9325}$, r^2 = 0.999; tocainide (*S*)-(+) enantiomer: GX peak area ratio = 1720 (tocainide (*S*)-(+) enantiomer)$^{0.9435}$, r^2 = 0.999.

Tocainide concentration (μg/ml)				Relative errors (%)	
(*R*)-(—)		(*S*)-(+)		(*R*)-(—)	(*S*)-(+)
Calculated*	Observed**	Calculated*	Observed**		
0.0100	0.0103	0.0100	0.00945	+3.0	—5.5
0.0250	0.0248	0.0250	0.0238	—0.8	—4.6
0.0500	0.0510	0.0500	0.0575	+2.0	+15.0
0.125	0.116	0.125	0.122	—7.2	+2.0
0.250	0.253	0.250	0.257	+1.2	+3.0
0.500	0.505	0.500	0.495	+1.0	—1.0
1.25	1.24	1.25	1.24	—0.4	—0.4
2.50	2.56	2.50	2.43	+2.2	—2.6
Spiked samples					
0.250	0.267	0.000	0.000	+6.8	0.0
0.000	0.000	0.250	0.252	0.0	+0.8
0.250	0.253	0.125	0.127	+1.2	+1.6

*By dilution, in plasma.
**By chromatography using the least-squares power curves; mean of triplicate samples.

The MTPA derivatives appeared to be stable when stored at 4°C or —20°C for at least 48 h as there was no significant change in the absolute peak areas or TOC/GX peak area ratios (Table I). The effect of longer storage times was not investigated systematically, but reassay of several samples after a two-week storage period gave results essentially identical to those obtained after 48 h of storage.

Calibration data are given in Table II for the determination of the enantiomers of TOC. Over the wide concentration range the calibration data were curvilinear, and attempts to describe the data with linear least-squares regression resulted in regression lines with marked systematic deviation at low TOC concentrations. Concentration-dependent extraction efficiency, limiting derivatization reaction, and non-linear electron-capture detector response were all investigated as possible sources of non-linearity. Non-linear detector response was found to be the cause of the curvilinearity of the calibration data, as demonstrated in Fig. 3. Calibration data for each enantiomer were well fitted by least-squares power curves (r^2 = 0.999) and are presented in Table II. These curves were used to calculate the concentration of each enantiomer in unknown samples, as illustrated for spiked plasma samples in Table II.

Intra-assay coefficients of variation (C.V.) were 3.3 and 2.1% for the (*R*)-(—) enantiomer of TOC at concentrations of 0.125 and 1.25 μg/ml, respectively, and 3.4 and 2.4% for the (*S*)-(+) enantiomer at the same concentrations. Day-to-day

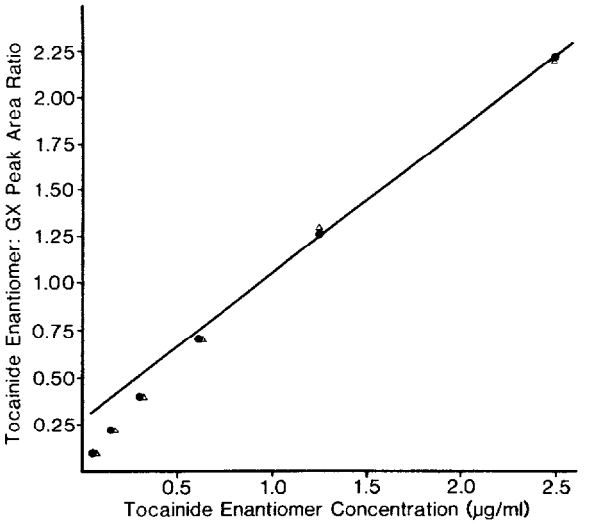
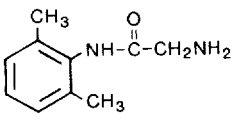
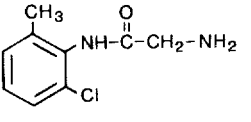


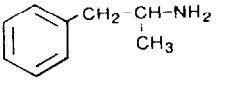
Fig. 3. Test of electron-capture detector response linearity. MTPA derivatives of (*R*)-(—)-TOC (•); (*S*)-(+)-TOC (△). The line was drawn arbitrarily to illustrate curvilinearity of the data.



GX



ACAT



Amphetamine

Fig. 4. Chemical structures of the internal standards and amphetamine.

C.V. values were 2.5 and 3.0% for the (*R*)-(—) and (*S*)-(+) enantiomers, respectively, at a concentration of 1.25 μg/ml. The lower limit of sensitivity of the assay for each enantiomer was found to be 10 ng/ml.

Derivatization of (*R,S*)-1-phenyl-2-aminopropane (amphetamine, Fig. 4) with (*S*)-MTPA-Cl and chromatography of the derivatives revealed that the response of the electron-capture detector to these derivatives is ca. 100-fold less than the response to the derivatives of tocainide.

The retention times of diazepam and nordiazepam were 8.1 and 12.8 min, respectively, under the chromatographic conditions used. The acid wash in the procedure removed 80% and 90% of diazepam and nordiazepam, respectively.

DISCUSSION

Several different approaches are available for the simultaneous determination of enantiomers in biological fluids [7]. We selected derivatization with a chiral

reagent and GLC separation of the resulting diastereomers for the analysis of the enantiomers of TOC. The chiral reagent chosen, (*S*)-MTPA-Cl, (Fig. 1) has been shown in our laboratory to be useful in the GLC resolution of TOC [3] and a variety of other chiral amines [5, 7, 8], and was used successfully in studies of the stereoselective disposition of TOC [3]. In these previous studies, flame ionization [5, 8] or nitrogen—phosphorus detection [3] was employed. The sensitivity provided by these detectors, however, was insufficient for studies of the pharmacokinetics of TOC in man. The observation that the chemical structure of the MTPA moiety includes the trifluoromethyl group (Fig. 1) suggested that the electron-capture detector would respond well to the MTPA derivatives. This expectation was realized, and excellent sensitivity was achieved in the assay of the enantiomers. Surprisingly, however, when the MTPA derivatives of amphetamine (Fig. 4) were chromatographed, the detector response was ca. 100-fold less than that for the TOC derivatives. It is clear that structural elements other than the trifluoromethyl group are also involved in the high response of the electron-capture detector to the MTPA derivatives of tocainide.

The use of MTPA-Cl for the resolution of the TOC enantiomers offers advantages over a recently published procedure [9] based on derivatization of TOC with heptafluorobutyric anhydride followed by GLC resolution of the derivatives on a glass capillary column coated with a chiral stationary phase. Both procedures include a derivatization step before chromatography, but the MTPA derivatives are resolved on a conventional (non-chiral) stationary phase in a packed column, a significant advantages over the use of chiral glass capillary columns in terms of ease of handling and cost. MTPA-Cl, having no exchangeable hydrogen at the chiral center, is stereochemically extremely stable, and the MTPA derivatives of TOC also have excellent chemical stability.

After considering several compounds, GX (Fig. 4) was selected to serve as the internal standard. GX is a primary amine with a chemical structure (Fig. 4) very similar to that of TOC (Fig. 1), and under the GLC conditions used, the retention time of its MTPA derivative is ideal (Fig. 2). Another advantage of GX, a non-chiral compound, is that it gives a single derivative with the chiral derivatizing agent. GX can be readily prepared in one step from commercially available starting materials. The response of the electron-capture detector to the MTPA derivative of GX is similar in magnitude to its response to the derivatives of TOC. GX is a metabolite of lidocaine [10], but this presents no problem in our pharmacokinetic studies, since in this controlled setting the absence of lidocaine in the study subjects is assured. If the assay procedure were to be used for the determination of TOC in samples from a patient receiving TOC and lidocaine simultaneously — an unlikely event — the use of GX as internal standard may result in spuriously low TOC concentrations. The presence in the sample of GX resulting from metabolism of lidocaine can be readily determined by processing the sample through the present procedure without the addition of internal standard. To circumvent the problem of the presence of lidocaine-derived GX in the sample, an alternative internal standard, ACAT (Fig. 4) may be used (data not shown). The only disadvantage of ACAT is a longer than optimum R_T (19 min). In all other respects, i.e., chemical structure, extractability, derivatization, electron-capture detector response, and com-

mercial availability, ACAT is well suited for the role of internal standard.

Since several benzodiazepines have been shown to elicit a high response from the electron-capture detector [11], potential interference from diazepam and nordiazepam was studied. It was found that extraction with 1 *M* hydrochloric acid, a step carried out after derivatization of TOC with MTPA-Cl, removes 80—90% of these benzodiazepines present in the ethyl acetate solution. The serum concentration of diazepam during chronic administration of therapeutic doses is in the range 0.7—1.5 μg/ml [12]. Since 80% of the amount present is removed during the assay procedure for TOC, and since the peak due to diazepam is adequately separated from the peaks of interest in the chromatogram, it is clear that this drug does not interfere with the analysis of TOC in the therapeutic range. If, however, low TOC concentrations (< 1 μg/ml) are measured in the presence of relatively high concentrations (> 1.5 μg/ml) of diazepam, interference by the latter drug may occur, inasmuch as the peak due to diazepam may partially overlap with the peak due to (*R*)-(—)-TOC. Serum concentrations of nordiazepam, the major circulating metabolite of diazepam, are in the range 35—52 ng/ml [12]. Since these already low concentrations are reduced by 90% during the assay procedure, nordiazepam does not interfere in the analysis, despite having a retention time nearly coinciding with that of GX. Other commonly used benzodiazepines, e.g., flurazepam, oxazepam and chlordiazepoxide, have retention times shorter than that of diazepam or longer than that of nordiazepam [11], and thus do not interfere with the determination of TOC. A previously published procedure did not evaluate potential interference from other drugs [9].

The chromatograms obtained display good peak shapes, and baseline resolution of the diastereomers was achieved (Fig. 2). The procedure provides high sensitivity and good accuracy and precision. The method was developed for the 1-ml sample size, but recent experiments in our laboratory (data not shown) indicate that smaller sample sizes (200—300 μl) may be used if necessary, with only a small loss of sensitivity (lower limit ca. 20 ng/ml). The procedure displays good selectivity as a result of several organic—aqueous partitioning steps, specific derivatization, selective detection, and chromatographic separation.

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