Journal of Chromatography, 221 (1980) 109—117
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 647

SIMULTANEOUS DETERMINATION OF *d*- AND *l*-PROPRANOLOL IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received March 20th, 1980; revised manuscript received June 1st, 1980)

SUMMARY

A method for the determination of *d*- and *l*-propranolol in human plasma is described. The method involves extraction of propranolol from plasma, and the formation of diastereomeric derivatives with the chiral reagent N-trifluoroacetyl-1-prolylchloride. Separation and quantitation of the diastereomeric propranolol derivatives are carried out by a reversed-phase high-performance liquid-chromatographic system with fluorimetric detection. The reproducibility in the determination of *d*- and *l*-propranolol in human plasma was 4.5% (relative standard deviation) at drug levels of 10 ng/ml.

In two subjects who received a single 40-mg tablet of racemic propranolol the plasma levels of the *d*-isomer were lower than of the *l*-propranolol. The half-lives of *d*- and *l*-propranolol were similar.

INTRODUCTION

The propranolol available for use as an adrenergic beta-receptor antagonist is a racemic mixture of two optical isomers, dextro (*d*) and laevo (*l*) propranolol. The *l*-isomer is more potent than the *d*-isomer and it is probably responsible for almost all the pharmacological effect [1, 2]. After administration of the racemate to animals the isomers have different disposition kinetics [3—5]. In man

0378-4347/80/0000-0000/$02.25

the half-life of *l*-propranolol was longer than that of *d*-propranolol after separate administration of the isomers [6]. However, data are lacking for the disposition of the isomers in man after administration of racemic propranolol. Such data are necessary especially in studies of concentration—effect relationships, since the concentration ratio between the two isomers may vary under different conditions and since only the *l*-isomer should contribute to the clinical effect.

This lack of human kinetic data on the *d*- and *l*-isomers of propranolol after administration of the racemate is due to the lack of suitable analytical methods. Previous methods used for separation of the isomers require sophisticated equipment and the use of isotopes [5, 7], immunological techniques of uncertain specificity [3], or gas—liquid chromatographic techniques which are not sensitive enough for studies of concentration—effect relationships in man [4].

This project was initiated to study the kinetics and effects of active *l*-propranolol in the presence of almost inactive *d*-propranolol after giving small doses of commercial racemic propranolol to man. To achieve this goal we developed a high-performance liquid chromatographic (HPLC) method which does not require complicated equipment nor isotopes but which is capable of quantitating the concentrations of *d*- and *l*-propranolol in plasma from subjects taking small doses of commercial propranolol. The method is based on the principles of forming diastereomeric derivatives with the chiral reagent N-trifluoroacetyl-1-prolyl chloride followed by separation of these propranolol derivatives on a reversed-phase column and fluorimetric detection.

MATERIALS AND METHODS

Chemicals

Racemic, dextro (*d*) and laevo (*l*) propranolol HCl were kindly supplied by Imperial Chemical Industries (Macclesfield, Great Britain). N-Trifluoroacetyl-1-prolyl chloride (N-TFA-1-prolyl chloride or TPC-reagent) 0.1 *M* in chloroform was obtained from Regis Chemical Co., Chicago, IL, U.S.A. Acetonitrile was of Grade S quality and purchased from Rathburn Chemicals, Walkerburn, Great Britain. LiChrosorb RP-8 and RP-18 were obtained from E. Merck, Darmstadt, G.F.R., and μBondapak phenyl from Waters Assoc. (Milford, MA, U.S.A.). All other reagents were of analytical or equivalent grade and used without further purification.

Apparatus

The pump was an Altex Model 100 solvent delivery system and the injector a Valco Model CV-6-UHPa (7000 p.s.i.). A Schoeffel FS 970 L.C. fluorimeter was used as the detector. It was operated with an excitation wavelength of 210 nm and a 340 nm cut-off emission filter. The mass spectrometer was an LKB 2091 equipped with a digital PDP 11/05 computer system and operated in the electron-impact mode at 70 eV.

Chromatographic technique

Chromatographic analyses were performed at room temperature. The mobile

phases were made with phosphate buffer (pH 2.2) with an ionic strength of 0.05. The mobile phases were degassed in an ultrasonic bath and brought to room temperature before use.

Column preparation

The column was made of 316 stainless-steel with a polished inner surface, equipped with modified Swagelok connections and Altex stainless frits (2 μm). The columns were 250 X 3.2 mm packed with either LiChrosorb RP-18, RP-8 with a mean particle diameter of 5 μm, or with μBondapak phenyl with a 10-μm particle size. The columns were packed by a modification of the ordinary balanced density slurry technique [8]. The supports were suspended in 15 ml of chloroform which was poured into the packing column. This was then filled with hexane and coupled to a Haskel pump. Acetone was used as driving liquid in the pump which was operated at 5000 p.s.i. After packing the columns were washed with 50 ml of acetonitrile and then with 100 ml of acetonitrile—water (50:50) before equilibration with the mobile phase consisting of 45% acetonitrile in phosphate buffer (pH 2.2) (μ = 0.05).

All columns were tested after packing in a test system with the mobile phase composition described above. *l*-Propranolol derivatized with the TPC reagent was used as solute for this test. Columns having a reduced plate height ($h = H/d_p$) of less than 10 at a flow-rate of 1 ml/min and an asymmetry factor of less than 1.5 were accepted for this study. The asymmetry factor was calculated by drawing a perpendicular to the base-line from the vertex formed by the two peak tangent lines. The back part of the peak base-line divided by the front part gives the asymmetry factor.

Extraction and derivatization of propranolol

One millilitre of 1 *M* carbonate buffer (pH 9.85) was added to 1.0-ml plasma samples containing propranolol. Propranolol was then extracted with 6.0 ml of water-saturated diethyl ether for 15 min. The tubes were centrifuged for 3 min at 4200 *g* and 5.0 ml of the ether layer were collected and evaporated at 40°C in a stream of dry nitrogen. The residue was dissolved in 0.5 ml of chloroform containing 25 μl of triethylamine (prepared just before use). One hundred microlitres of 0.1 *M* TPC reagent were added and reacted for 15 min at room temperature (see below). The reaction was stopped by the addition of 3.0 ml of water and 6.0 ml of water-saturated ether to two tubes at a time. After extraction for 2 min the tubes were centrifuged for 3 min at 4200 *g*. A 5-ml aliquot of the ether layer was evaporated to dryness at 40°C under nitrogen. The samples were dissolved in 170 μl of mobile phase and 150 μl were injected onto the column. The reaction time needed to obtain maximum yield of the propranolol derivatives was determined using six standard solutions containing each propranolol isomer at 25 ng/ml. The reaction was stopped in two tubes at a time at 2, 15 and 30 min, respectively.

Purity of propranolol enantiomers

The optical purity of *d*- and *l*-propranolol were determined by chromatography of the enantiomers in underivatized form in an ion-pair HPLC system using LiChrosorb Diol as support and a mobile phase consisting of dichloromethane, 1-pentanol with *d*-camphersulfonic acid as the counterion [9].

Standard curves

Two sets of six samples containing 1, 5, 10, 25, 40 and 50 ng/ml each of *d*- and *l*-propranolol hydrochloride in 1 *M* carbonate buffer (pH 9.85) were added to 1.0 ml of drug-free plasma. Extraction and derivatization were performed as described above. Peak heights were measured and plotted against the concentration of *d*- and *l*-propranolol.

Reproducibility studies

Reproducibility studies for analysis of *d*- and *l*-propranolol were performed at four different concentrations (1, 10, 25 and 50 ng/ml) of *d*- and *l*-propranolol hydrochloride dissolved in 1 *M* carbonate buffer (pH 9.85). Five 1.0-ml samples at each concentration were added to 1.0 ml of drug-free plasma and analyzed according to the procedure described above and the relative standard deviations were calculated.

Identification of the derivative

Eluted peaks were collected from the HPLC column to confirm the identity of the propranolol derivatives. The acetonitrile was evaporated and the derivative was extracted with ether. The ether was evaporated and the residue redissolved in chloroform; an aliquot was injected directly into the ion source of the mass spectrometer.

Subjects

After fasting for 8 h, two healthy male volunteers (37 years old) each took a single commercial Inderal® (ICI) tablet containing 40 mg of racemic propranolol. At timed intervals venous blood was drawn into heparinized Venoject tubes according to a protocol approved by our Ethical Committee. Plasma was separated immediately after collection and frozen until analyzed.

RESULTS AND DISCUSSION

Extraction procedure

The acid dissociation constant (pK_a) for the secondary amino function of propranolol is 9.5 [10]. Plasma samples and standard solutions were adjusted to pH 9.85 by 1 *M* carbonate buffer. This gave > 98.0% extraction of propranolol into diethyl ether with a phase volume ratio (V_{org}/V_{aq}) of 2.5.

Derivatization of propranolol

Derivatization of enantiomers with an optically active reagent yields diastereomers which often can be separated by HPLC [11] or gas—liquid chromatography [12]. In this assay procedure an HPLC system was used to separate and quantitate the diastereomeric derivatives formed by reaction of propranolol in human plasma with the chiral reagent N-trifluoroacetyl-l-prolyl chloride (TPC). The utility of this reagent as a resolving agent for enantiomers was demonstrated by Weygand et al. [13, 14]. Mass spectrometry was used to confirm the identity of the diastereomeric derivatives. The mass spectra of the diastereomers were essentially identical (Fig. 1). The fragmentation pattern coincided with that for the trimethylsilyl derivative of TPC-propranolol [12].

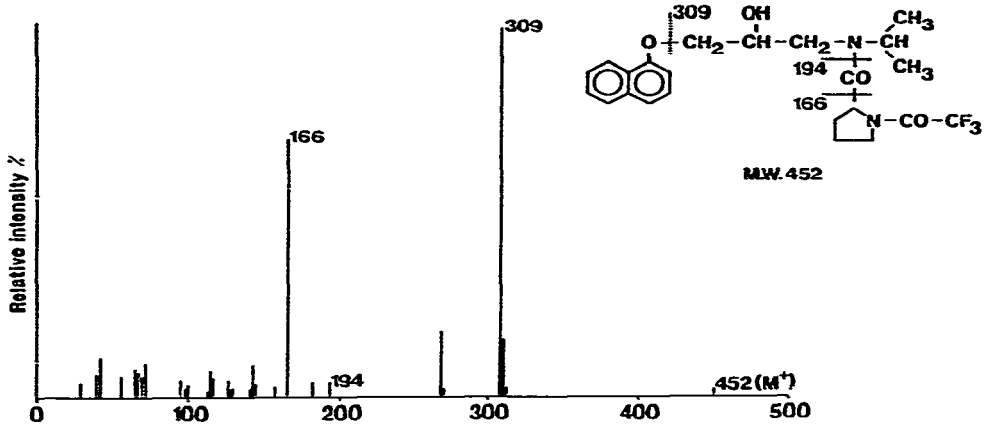

Fig. 1. Mass spectrum of N-trifluoroacetyl-1-prolyl chloride propranolol derivative.

The reaction time needed to obtain maximum yield was 15 min. No decrease in the yield was observed after 30 min of reaction time which indicates that no reaction takes place at the hydroxy function of propranolol during the time period studied. The reaction mixture was cleaned up by extraction with 3.0 ml of water and 6.0 ml of ether.

Chromatographic studies

A reversed-phase liquid chromatographic system was used to separate the diastereomers formed by reaction of propranolol with the chiral reagent. Several types of stationary phases (μBondapak phenyl, LiChrosorb RP-8 and RP-18) were tested to find which gave the highest selectivity for the diastereomers. LiChrosorb RP-18 gave the best selectivity with an α value of 1.2 with a mobile phase composition of 45% acetonitrile in phosphate buffer at pH 2.2 and a flow-rate of 1.0 ml/min. With this mobile phase the *l*-propranolol derivative had a capacity factor of 11.9 and the *d*-propranolol derivative a capacity factor of 14.4. The peaks of the *l*- and *d*-propranolol derivatives have asymmetry factors of 1.3 and 0.9, respectively.

A small increase in the asymmetry factor and the H value of the two diastereomeric derivatives have been observed over a long period of use, but after four months' use the H values of the *l*- and *d*-propranolol derivatives were 0.05 and 0.08, respectively. Fig. 2A shows a separation of the diastereomeric propranolol derivatives from human plasma using a four-month-old column. There were no disturbances of endogenous substances in plasma but four unexplained negative peaks can be seen in chromatograms of standard and plasma samples (Fig. 2B).

Quantitation of d-TFA-prolyl chloride in the reagent

Because commercial TPC-reagent contains small amounts of the *d*-isomer, four diastereomeric derivatives are formed upon reaction with racemic propranolol. Chromatographic experiments showed that *l*-propranolol derivatized with the TPC-reagent gave one main peak and a minor peak constituting 8.5 ± 0.2% (n = 5) of the main peak eluted with the same capacity factor as *d*-propranolol 1-TFA proline. *d*-Propranolol derivatized in the same way gave an equal

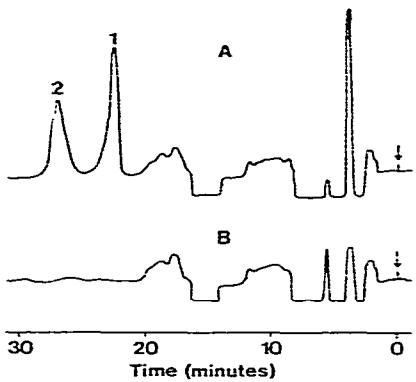

Fig. 2. Chromatograms of (A) a plasma sample containing *l*-propranolol (1) at 12.3 ng/ml and *d*-propranolol (2) at 8.9 ng/ml, and (B) of a blank plasma sample.

peak with the same capacity factor as *l*-propranolol 1-TFA proline. A likely cause of the minor peaks is that the *d*-TFA-proline fraction of the reagent forms *l*-propranolol *d*-TFA proline and *d*-propranolol *d*-TFA proline. This is supported by the fact that the manufacturer reports 8.2% of *d*-TFA prolyl chloride in the reagent which is in accordance with the above results. This may also indicate that no racemization takes place during the derivatization of propranolol. To confirm that the minor peaks were reaction products of the propranolol enantiomers and *d*-TFA-prolyl chloride, a racemic TPC reagent was prepared according to the description of Bonner [15]. Reaction of a solution of *d*- and *l*-propranolol (100 ng/ml) separately with the racemic reagent gave two peaks in each chromatogram. The peak heights were measured and the concentration in each peak was calculated from the standard curve and was found to be 50 ng/ml. A mixture of equal amounts of the two reaction mixtures gives only two peaks in the chromatogram with the same concentration and with the same capacity factors as obtained for racemic propranolol derivatized with the commercial TPC-reagent. The above experiment was performed with *d*- and *l*-propranolol each with an enantiomeric purity of > 99.5%. The optical purity of the propranolol enantiomers was checked as described in Materials and methods.

Correction of the results due to contamination of the TPC-reagent

Because the TPC-reagent contained *d*-TFA-prolyl chloride it was necessary to correct the concentrations obtained from peak height measurements according to the following equations

$$X = X_{\text{Chrom}} + AX - AY \quad (1)$$

$$Y = Y_{\text{Chrom}} + AY - AX \quad (2)$$

where X and Y represent the concentrations of *d*- and *l*-propranolol, respectively, that would be obtained if no *d*-TFA-prolyl chloride were present in the TPC-reagent. X_{Chrom} and Y_{Chrom} are the concentrations calculated from the peak heights in the chromatograms of *l*- and *d*-propranolol, respectively, when working with a contaminated reagent with respect to *d*-TFA-prolyl chloride, and A

is the percentage of *d*-TFA-prolyl chloride in the reagent. From eqns. 1 and 2 it can be seen that when TPC-reagent contaminated with *d*-TFA-prolyl chloride reacts with racemic propranolol, no correction of the results obtained has to be made because AX is in this case equal to AY. Eqns. 1 and 2 then reduce to $X = X_{Chrom}$ and $Y = Y_{Chrom}$, respectively. However, the situation is different when the concentration ratio of *d*-propranolol/*l*-propranolol is smaller or larger than unity, as is the case in patients' plasma. Combination of eqns. 1 and 2 gives

$$Y = \frac{Y_{Chrom}(1 - A)}{1 - 2A} - \frac{AX_{Chrom}}{1 - 2A} \quad (3)$$

where $\frac{1 - A}{1 - 2A} = K_1$ and $\frac{A}{1 - 2A} = K_2$. In this case the *d*-TFA-prolyl chloride content in the reagent is 8.5% ($A = 0.085$), giving $K_1 = 1.1$ and $K_2 = 0.1$ which means that

$$Y = 1.1\ Y_{Chrom} - 0.1\ X_{Chrom} \quad (4)$$

In analogy

$$X = 1.1\ X_{Chrom} - 0.1\ Y_{Chrom} \quad (5)$$

Optimization of the fluorimetric response

The method is sensitive enough to quantitate therapeutic concentrations of the two propranolol enantiomers in human plasma. The high sensitivity with this method is largely the result of using 210 nm as the excitation wavelength which we have found to give optimal fluorimetric response. Other investigators working with the quantitation of propranolol use an excitation wavelength of 295 nm, which gives only 6% of the response obtained with an excitation wavelength of 210 nm [16, 17] and a 340 nm cut-off emission filter. Further reasons for the high sensitivity obtained with our method are the small internal diameter of the column (3.2 mm) giving rise to a small area of cross-section and interstitial volume [18], and the optimal geometry and small volume (5 μl) of the flow-cell of the detector.

Standard curve and reproducibility studies

The reproducibility of the method was determined as described under Materials and Methods. The study was performed at four different concentrations (1, 10, 25 and 50 ng/ml) of *d*- and *l*-propranolol hydrochloride and the relative standard deviations found were 5.9, 4.5, 2.8 and 2.4%, respectively. Standard samples were extracted after the addition of 1.0 ml of drug-free plasma, which has the advantage of giving the same degree of propranolol extraction from standard and plasma samples thus making compensation due to different recoveries unnecessary.

A standard curve is constructed by plotting the peak height against the concentration of each enentiomer. Linear regression equations of the standard curve for *l*- and *d*-propranolol were calculated and are given below.

$Y_l = 5.634\ x + 0.3396$
$Y_d = 4.428\ x + 0.8428$

The linearity of the standard curves in the range 1.0—45 ng of base per ml for each enantiomer was determined by calculating the correlation coefficients which were found to be 0.9994 and 0.9991 for the *l*- and *d*-propranolol derivatives, respectively.

Pharmacokinetics

The plasma concentrations of *d*- and *l*-propranolol measured in two subjects who received a single, oral 40-mg dose of racemic propranolol are shown in Fig. 3. After half an hour the concentrations of *d*- and *l*-propranolol were similar in one subject (Fig. 3A). Later the concentration of *d*-propranolol was always lower than that of the *l*-isomer. The plasma half-lives were similar for the enantiomers, 3.4 h. In the other subject (Fig. 3B), the *d*-propranolol concentration was lower at all time points. Thus the area under the plasma concentration—time curve (AUC) was lower for *d*- than for *l*-propranolol after a 40-mg oral dose (racemate). Also in this subject the half-lives were the same, 3.2 h. These data are in agreement with the report that the plasma concentration of *d*-propranolol is lower than that of *l*-propranolol for up to 1.5 h after giving the separate isomers orally [6] and also with in vitro human liver data where we have shown that *d*-propranolol is more rapidly oxidized than *l*-propranolol [19]. These data disagree with findings in dogs, where the AUC in plasma was lower for the *l*-isomer [5]. We have no definite explanation of this discrepancy, and we have only studied two subjects. However, the data may indicate a species difference with regard to the metabolism of propranolol between dog and man.

In this study the plasma half-lives of *d*- and *l*-propanolol were similar after oral administration of racemic propranolol (Fig. 3). This is consistent with theories on hepatic elimination of "high-clearance" drugs [20] since both *d*- and *l*-propranolol are highly extracted by the liver. George et al. [6] found that the half-life of *d*-propranolol was shorter than that of *l*-propanolol when the isomers were given separately. This may be the result of reduced hepatic blood flow caused by *l*- but not *d*-propranolol [21]. Decreased hepatic blood flow should prolong the half-life of a "high-clearance" drug like propranolol. Our

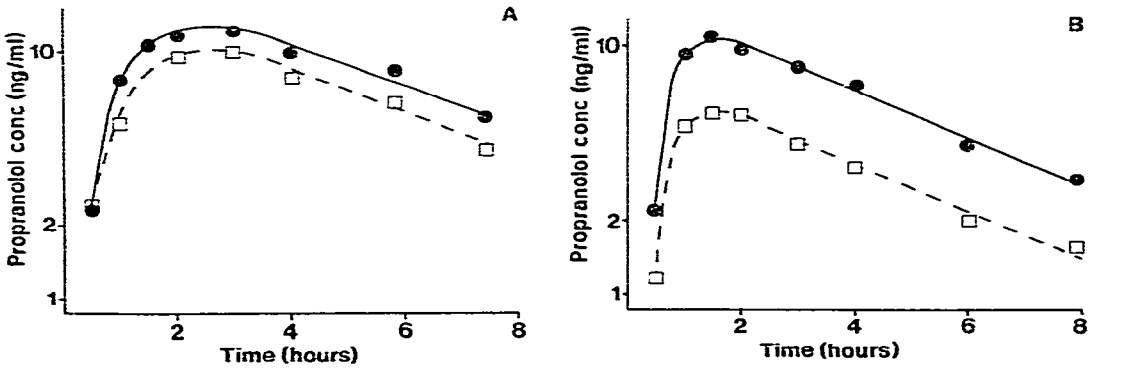

Fig. 3. Plasma concentrations of *d*-propranolol (□ --- □) and *l*-propranolol (● — ●) in two subjects (A and B) receiving 40-mg commercial tablets containing racemic propranolol. Values are corrected according to eqns. 4 and 5.

data and those of George et al. [6] are not inconsistent since after administration of the racemate the clearance of both *d*- and *l*-propranolol would be affected by the decreased liver blood flow caused by *l*-propranolol. However, if only *d*-propranolol is given, the liver blood flow should not be decreased and thus the plasma half-life would be shorter.

In conclusion, a high-performance liquid chromatographic method was developed by which both *d*- and *l*-propranolol can be separated and quantitated separately in clinical plasma samples after administration of small doses of racemic propranolol. The method requires no complicated techniques or the use of isotopes. We use this method in human pharmacodynamic studies.

ACKNOWLEDGEMENTS

This work was supported by the Swedish Medical Research Council (14X-05677; 04X-03902), Funds from the Karolinska Institute, Loo Osterman and Swedish National Association against Heart and Chest Diseases. We are grateful to Professor G. Schill for valuable discussions of the manuscript.

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