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ENANTIOSELECTIVE DRUG MONITORING OF (*R*)- AND (*S*)-PROPRANOLOL IN HUMAN PLASMA VIA DERIVATIZATION WITH OPTICALLY ACTIVE (*R,R*)-O,O-DIACETYL TARTARIC ACID ANHYDRIDE

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SUMMARY

A sensitive high-performance liquid chromatographic method was developed for the stereoselective assay of (*R*)- and (*S*)-propranolol in human plasma. The method involves diethyl ether extraction of the drugs and a racemic internal standard, N-*tert.*-butylpropranolol, followed by derivatization of the compounds with the chiral reagent (*R,R*)-O,O-diacetyl tartaric acid anhydride. The resulting diastereomeric derivatives were separated isocratically on a reversed-phase column. Quantitation was achieved by the peak-height ratio method with reference to the internal standard. The assay was accurate and reproducible in the concentration range 1–100 ng of (*R*)- and (*S*)-propranolol per ml plasma, using fluorescence detection at λ_{ex} 290 nm and λ_{em} 335 nm. The applicability of this method was demonstrated for the determination of concentration–time profiles of propranolol enantiomers in the course of comparative pharmacokinetic studies.

INTRODUCTION

For about twenty years the β-receptor-blocking drug propranolol has been clinically applied as a racemic mixture consisting of 50% (*R*)- and 50% (*S*)-propranolol. However, it has been shown that the β-adrenergic activity of the drug is due to the (*S*)-enantiomer which is about 100 times more active than (*R*)-propranolol [1–3]. In addition to their different pharmacodynamic profiles, (*R*)-

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and (*S*)-propranolol may also differ in other stereoselective controlled processes such as drug absorption, distribution, metabolism and elimination. These processes have been studied extensively by Walle et al. [4].

Recently, Ariens et al. [5] have appealed to medical chemists, pharmacokineticists and clinical pharmacologists to pay particular attention to the stereochemical aspects and biological activity of xenobiotics. Techniques for the enantioselective monitoring of drugs (and metabolites) are necessary for the accomplishment of this task.

Several analytical approaches to the resolution and monitoring of propranolol isomers in biological fluids have been published. Methods for the monitoring of other β-blockers have also been reported. Walle and Walle [6] have recently reviewed three techniques that have been applied successfully in this field: the chromatographic, the biological and the stable-isotope methods. Applications using liquid chromatography for enantioseparation have become quite popular [7,8], especially the so-called indirect technique [9], which is based on derivatization of the chiral analytes (in our case the β-blockers (*R*)- and (*S*)-propranolol) with an optically pure chiral derivatizing reagent (CDR) to form a pair of diastereomeric derivatives, which are theoretically separable on non-chiral HPLC systems.

β-Blockers have an aminoalcohol structure with a secondary hydroxy and a secondary amine function accessible for derivatization. As summarized in Table I, a number of the CDRs (entries 1–6) have been applied to the direct resolution of aminoalcohol enantiomers by attacking the amine and converting it into an amido function. The resolution factors *R* (Table I) of the corresponding pair of diastereomeric amido derivatives are sufficient for quantification as long as the peak-size ratios do not exceed 10:1, but it may be a problem to quantify peak-size ratios of 100:1 or less. We have taken another approach by using (*R,R*)-O,O-diacetyl tartaric acid anhydride (DATAAN) as the CDR [10]. This reagent forms tartaric acid monoesters of aminoalcohols (see Fig. 1). With DATAAN (entry 7 in Table I) resolution factors between 4 and 6 could be obtained easily, owing to additional intramolecular ion-pair bonding (Fig. 1).

TABLE I

OPTICALLY ACTIVE REAGENTS USED TO CONVERT β-BLOCKERS INTO DIASTEREOMERIC DERIVATIVES

CP = chromophor, FP = fluorophor; HPLC systems: predominantly reversed-phase.

No.	Reagent	Abbreviation	Chromophor	Resolution	α Value	Ref.
1	(−)-N-Trifluoroacetylprolyl chloride	(−)-TPC	–	1.4	1.2	11–13
2	t-Boc-L-Leu anhydride		–	1.7	1.3	14, 15
3	(+)- and (−)-Phenyl isocyanate	(+)-PEI (−)-PEI	CP	1.5–2.6	1.1 1.25	16–18 19–21
4	(*R*)-(−)-1-(1-Naphthyl)ethyl isocyanate	(−)-NEI	FP	1.5	1.1	22, 23
5	2,3,4,6-Tetra-O-acetyl-β-D--glucopyranosyl isothiocyanate	TAGIT	–	2.7	1.6	24–26
6	(+)-1-(9-Fluorenyl)ethyl chloroformate	FLEC	FP	1.2	1.06	27
7	(*R,R*)-O,O-Diacetyl tartaric acid anhydride	DATAAN	–	5.0	2.7	10

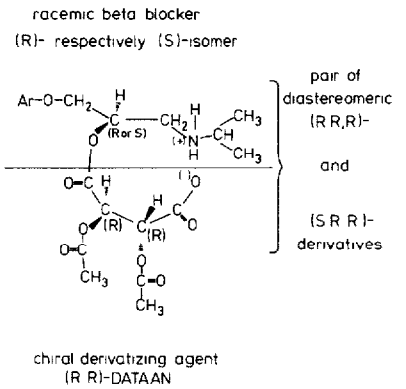

Fig. 1. Structures of the diastereomeric (*R,R*)-O,O-diacetyl tartaric acid monoesters of (*R*)- and (*S*)-propranolol (Ar = 1-naphthyl).

This paper describes the adaptation of this approach to biological samples. This method is capable of determining the plasma profiles of the (*R*)- and (*S*)-isomers in subjects who received single oral doses of either (*S*)- or (*R,S*)-propranolol.

EXPERIMENTAL

Chemicals

Racemic (*R,S*)-propranolol hydrochloride (USP quality) was supplied by Schweizerhall (Basel, Switzerland). The preparation of optically pure (*R*)- and (*S*)-propranolol hydrochloride was performed according to a method developed by Lindner [28]; the chemical purity of the drugs was >99.8% and the optical purity (o.p.) of (*R*)-propranolol was >99.3% and of (*S*)-propranolol 99%, as analysed by an indirect enantioselective high-performance liquid chromatographic (HPLC) technique [10].

The internal standard, racemic (*R,S*)-1-*tert.*-butylamino-3-(1-naphthyloxy)-2-propanol [(*R,S*)-*tert.*-butylpropranolol] was synthesized according to the following procedure. To a solution of 2.9 g (20 mmol) of 1-naphthol in 30 ml (390 mmol) of epichlorohydrine were added 4.4 g (ca. 22 mequiv. OH^-) of ion-exchange resin (III, Merck) and the mixture was refluxed for 4 h [29]. After filtration and evaporation of the excess reagent the residue was twice taken up in toluene and evaporated again. The crude (*R,S*)-1-(2,3-epoxypropoxy)-naphthalene was taken up in hot petroleum ether, the solvent was evaporated, and the residue (3 g) was refluxed with 30 ml (287 mmol) of *tert.*-butylamine for 16 h. The excess reagent was evaporated and the residue was taken up in diethyl ether (30 ml) and washed with water (two 15-ml portions), and ca. 4.5 ml of 4 *M* hydrochloric acid were added (pH 4.3). The ethereal phase was removed, and the hydrochloride of the (*R,S*)-*tert.*-butylpropranolol [30] crystallized after several hours. The yield was 2.5 g (40%) after recrystallization from water, and the m.p.

was 180°C. The empirical formula was $C_{17}H_{23}NO_2 \cdot HCl$, and the NMR data ($CDCl_3$, free base) were: δ 8.3–6.8 (m, naphthyl); 4.12 (m, CH_2O, CHO); 2.80 (m, CH_2N, OH, NH); 1.10 (CH_3) ppm.

The internal standard has the same molar extinction coefficient at 290 nm as propranolol (256 000 in methanol). The (*R*)- and (*S*)-forms of propranolol and the internal standard are enantiomers and show similar physico-chemical behaviour. This is not true for diastereoisomers, in this case the diastereomeric tartaric acid derivatives (see Fig. 1). With UV detection ($\lambda = 290$ nm) the peak areas of equal amounts of (*R*)- and (*S*)-propranolol derivatives differ by a factor of 1.08. With fluorescence detection (λ_{ex} 290 nm, λ_{em} 335 nm) an even stronger difference was observed. The later eluting peak of the corresponding pair (the derivative with (*S,R,R*)-configuration, see also formula in Fig. 1) was 20% less fluorescence-active than the corresponding (*R,R,R*)-derivative. The same was found for the internal standard derivatives (see Fig. 2B).

Trichloroacetic acid, DATAAN, dichloromethane (DCM), diethyl ether and methanol p.a. grade) were purchased from Fluka (Buchs, Switzerland). The HPLC-grade solvents were obtained from Loba (Fishamend, Austria). The Extrelut® columns and the refilling material were from Merck (Darmstadt, F.R.G.).

The dosage form of the drugs (hard gelatin capsules), containing 80 mg of (*R,S*)-, 40 mg each of (*R*)- and (*S*)-propranolol, and *D*-mannitol and carbosil as auxiliary materials, was produced according to specifications of the European Pharmacopoeia.

Apparatus

The HPLC system comprised a 110A pump (Altex), a Model 3000 fluorescence detector (Perkin-Elmer), a Model 7120 injector, a 50-μl loop (Rheodyne) and a Model 3390A integrator (Hewlett-Packard). A 125 mm×4 mm I.D. column packed with 5-μm C_{18} Hypersil (Seibersdorf, Austria), together with a pre-column (15 mm×4 mm I.D.) packed with Lichrosorb RP_{18} (10 μm, Brownlee Labs., Santa Clara, CA, U.S.A.) were used. The mobile phase was 2% aqueous acetic acid–acetonitrile (30:70) adjusted to pH 4.0 (apparent pH value) with concentrated ammonia and used at a flow-rate of 1 ml/min. The fluorescence detector was set to λ_{ex} 290 nm and λ_{em} 335 nm.

Extraction and derivatization procedure

The pharmacokinetic study was approved by an ethic committee and informed consent was given by all volunteers. The plasma samples obtained from the subjects were stored frozen at −20°C until analysis. For method development, pooled human plasma from a hospital was used.

To a 1-ml sample of thawed plasma, 1 ml of 0.5 *N* sodium hydrogencarbonate–sodium carbonate buffer (pH 10) and 100 μl of internal standard solution (300 ng/ml) were added and vortex-mixed for 10 s. The sample was placed on the top of a laboratory-made Extrelut extraction column (15 cm×1.4 cm I.D.) packed with a 2.5-cm layer of anhydrous sodium sulphate as drying agent at the bottom and on the top with a 4.5-cm layer of Extrelut material. The aqueous stationary phase adsorbed on the Extrelut material was extracted with 15 ml of diethyl ether and collected in a conical tube to which 100 μl of a solution of 0.01 *M* trichloro-

acetic acid in dry DCM and 100 μl of a 0.25 *M* solution of DATAAN in acetic acid–DCM (20:80) were added and mixed. The stoppered tubes were incubated for 4 h at 40°C, then the organic solvent was blown off by nitrogen. The residue was washed of the tube walls with 1 ml of methanol, which was also evaporated with nitrogen. By this step all excess reagent was quenched; the resulting methyl ester did not interfere in the HPLC analysis. The residue was redissolved in 20 μl of acetic acid, followed by 20 μl of methanol and 60 μl of water; 50 μl of this solution were injected into the HPLC column.

Extraction effectiveness

Six blank samples of pooled serum (1 ml) were spiked with 100 ng of racemic propranolol and internal standard. The samples were extracted, evaporated and treated as described but without adding the derivatizing agent DATAAN. The extracts containing propranolol and the internal standard were analysed on a reversed-phase HPLC system [column, 125 mm×4 mm I.D., Hypersil ODS 5 μm; mobile phase, methanol–2% acetic acid (55:45) adjusted to a pH of 4.8 with concentrated ammonia] and the absolute peak areas of the drugs were compared with aqueous external standard solutions (1 μg/ml each).

Standard curves and reproducibility

To aqueous samples as well as to blank pool plasma samples (1.0 ml) 4, 10, 20, 40, 60 and 80 ng of racemic propranolol hydrochloride and 30 ng of racemic internal standard were added by using appropriate volumes of stock solutions. The aqueous samples were analysed (extracted and derivatized) by the complete assay. Standard curves were drawn by calculating peak-height ratios of the derivatives of (*R*)-propranolol and (*R*)-internal standard as well as of (*S*)-propranolol and the (*S*)-isomer of the internal standard. In order to check the reproducibility of the assay the 10- and the 80-ng samples were analysed six times each.

Protocol for pharmacokinetic investigation

The study was performed in a randomized, double-blind, cross-over mode with eight healthy, fasted volunteers (five males, three females). In an interval of one week each subject was given orally either 80 mg of (*R,S*)-propranolol or 40 mg of (*S*)-propranolol. Starting with the drug application (zero time), blood samples were taken according to the time schedule (see Fig. 3A and B). The blood samples were centrifuged, frozen at −20°C and stored till analysis. In this paper the results of one subject are shown to illustrate the applicability of the assay.

RESULTS AND DISCUSSION

Chromatography

With authentic samples of the DATAAN derivatives of (*R*)- and (*S*)-propranolol [28] (e.g. (*R,R*)-O,O-diacetyl tartaric acid monoester of (*R*)-propranolol) as well as of the internal standard, the retention times and the elution order, respectively, have been examined in the chromatogram. At the given mobile phase conditions the separation factor α for the propranolol isomers was 1.84 and for

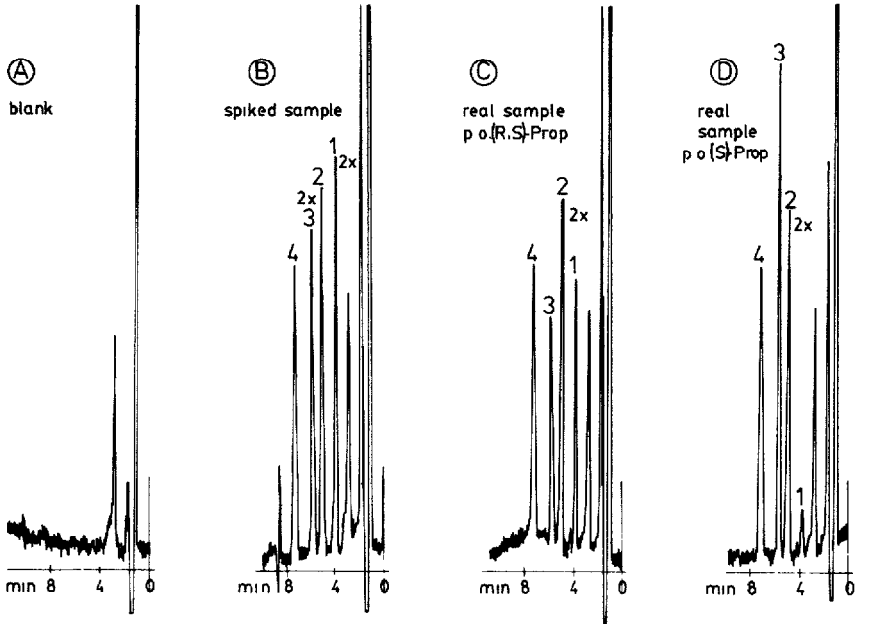

Fig. 2. Typical chromatograms of derivatized propranolol and internal standard isomers. (A) Blank human plasma; (B) human plasma spiked with 30 ng of racemic drugs, (*R,S*)-propranolol and (*R,S*)-internal standard; (C) human plasma 2 h after oral administration of 80 mg of (*R,S*)-propranolol (analysis values: 5 ng of (*R*)- and 11 ng of (*S*)-propranolol); (D) human plasma 2 h after oral administration of 40 mg of (*S*)-propranolol (analysis value: 20 ng of (*S*)-propranolol). Peaks: 1 = (*R*)-propranolol; 2 = (*R*)-internal standard; 3 = (*S*)-propranolol; 4 = (*S*)-internal standard derivative. HPLC conditions: column, 125 mm × 4 mm I.D. packed with Hypersil ODS; mobile phase, 2% acetic acid–acetonitrile (30:70) (apparent pH 4.0, adjusted with concentrated ammonia); flow-rate, 1 ml/min; fluorescence detection, λ_{ex} 290 nm and λ_{em} 335 nm.

the internal standard 1.64; the corresponding resolution factors were 4.0 and 3.8. There is only limited peak asymmetry noticable ($As = 1.1$), which is most probably due to the intramolecular ion-pair formation (see Fig. 1) masking the amine functionality that is usually responsible for pronounced peak asymmetry. Typical chromatograms of blank plasma and samples from subjects who received (*R,S*)- or (*S*)-propranolol are shown in Fig. 2. About a dozen different drugs that might be co-administered with propranolol during clinical therapy were checked for possible interferences in the chromatography; in all cases the chromatograms were clean in the relevant retention window.

Extraction and derivatization yield

Recoveries of racemic (*R,S*)-propranolol and racemic internal standard from spiked water and plasma samples were 68 ± 4.5% (4 ng/ml, $n = 6$) and 72 ± 2.7% (80 ng/ml, $n = 6$) for both matrices. The extractabilities were equal for propranolol and the internal standard in the total assay without the addition of the derivatizing agent (DATAAN). The derivatization yield was found to be 97 ± 5% ($n = 6$) in the high concentration range and 93 ± 7% ($n = 6$) in the lower concentration range. The molar excess of DATAAN to the drugs is high (more than 100 times), but no serious side-reactions have been observed. However, the presence

of water has to be avoided to ensure quantitative derivatization reactions and reliable results.

Owing to the quantitative reaction yield there is no risk or error based on possible different reaction kinetics of the enantiomers with DATAAN (kinetic resolution) [10]. The finally redissolved samples are modestly stable in this solution; slow hydrolysis takes place with the consequence that the redissolved sample extracts should be analysed within 2 h.

Standard curves and reproducibility

For the assay of human plasma spiked with racemic (*R,S*)-propranolol hydrochloride in the concentration range 4–100 ng/ml (six-point standard curves), coefficients of correlation for (*R*)- and (*S*)-propranolol of 0.996 and 0.995, respectively, were observed. Typical equations of the lines were: for (*R*)-propranolol, $y=0.806x-0.082$; for (*S*)-propranolol, $y=0.82x+0.084$. Occasionally a small interference peak in the blank samples coeluting with (*R*)-propranolol has been noticed, corresponding to a maximum value of ca. 0.3 ng/ml. The limit of determination was found to be 0.5 ± 0.3 and 1 ± 0.4 ng/ml for (*R*)- and (*S*)-propranolol, respectively.

The day-to-day reproducibility of peak-height ratios was sufficient (coefficient of variation = 4.7% for (*R*)-propranolol and the (*R*)-isomer of the internal standard and 6.5% for (*S*)-propranolol and the (*S*)-isomer of the internal standard). In routine analysis we inserted two replicated standard samples of blank plasma spiked with 30 ng of (*S*)- and 60 ng of (*R,S*)-propranolol and 30 ng of racemic internal standard every twenty samples. The intra-day reproducibility for triplicate samples of two subjects is summarized in Table II.

Application

The enantioselective assay was applied to a pharmacokinetic study in humans. Plasma levels of the propranolol enantiomers were determined at the following times: 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 9 and 12 h after per oral application of 40 mg of (*S*)- or 80 mg of (*R,S*)-propranolol. Rather untypical plasma concentration–time curves from one subject are shown in Fig. 3A and B. Detailed results of this study will be presented elsewhere [31]. However, it should be mentioned, that in

TABLE II

REPRODUCIBILITY OF PLASMA ASSAY

Plasma levels 2 h after oral administration.

Subject	Oral dose	(*R*)-Propranolol (ng/ml)			Mean	(*S*)-Propranolol (ng/ml)			Mean
2	80 mg (*R,S*)-propranolol	80	84	81	82 ± 2.1	105	111	102	106 ± 4.5
4	40 mg (*S*)-propranolol					37	32	34	34 ± 2.5

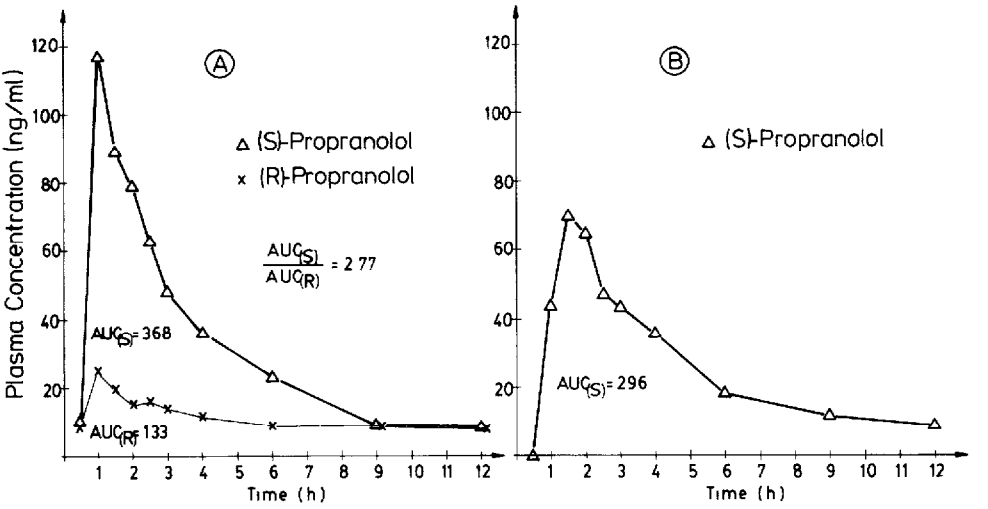

Fig. 3. Plasma concentration–time profiles for (*R*)- and (*S*)-propranolol (A) Profiles for subject No. 8 receiving 80 mg of (*R,S*)-propranolol orally. (B) Profile for the same subject receiving 40 mg of (*S*)-propranolol orally.

this present case the quotient of the areas under the concentration–time curves for (*R*)- and (*S*)-propranolol was 2.77, and rather different from the mean value, which is ca. 1.5 [31] and which is in agreement with previous studies in humans [4,32].

CONCLUSION

The study confirms that the indirect enantioseparation of aminoalcohols using DATAAN as the chiral derivatizing reagent is applicable to extracts of biological samples. In a comparative pharmacokinetic study with (*R,S*)- and (*S*)-propranolol, it was demonstrated that the enantioselective HPLC assay is sufficiently sensitive and reliable to monitor (*R*)- and (*S*)-propranolol down to ca. 1 ng/ml plasma per isomer. For the first time an isomer ratio of 200:1 could easily be analysed, which might be necessary to detect traces of the minor isomer in biological samples of optically active aminoalcohol-type drugs. This assay has been successfully applied for pharmacokinetic studies analysing the enantiomer ratio and time profiles of (*R*)- and (*S*)-propranolol in human plasma.

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