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SIMULTANEOUS DETERMINATION OF (*R*)- AND (*S*)-CELIPROLOL IN HUMAN PLASMA AND URINE: HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY ON A CHIRAL STATIONARY PHASE WITH FLUORIMETRIC DETECTION[a]

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SUMMARY

The quantitative enantiospecific determination of the β_1-selective adrenergic antagonist (*R,S*)-celiprolol in human plasma and urine is described It involves a two-step liquid–liquid extraction of celiprolol from biological material and separation of the underivatized enantiomers by high-performance liquid chromatography on a chiral stationary phase (cellulose tris-3,5-dimethylphenyl carbamate, coated on silica gel) with fluorimetric detection *R*-(+)-Propranolol was used as an internal standard The detection limits of 1 5 ng/ml enantiomer in plasma and 2 5 ng/ml enantiomer in urine at signal-to-noise ratios higher than 3 permit the performance of pharmacokinetic studies after therapeutic doses

INTRODUCTION

(*R,S*)-Celiprolol hydrochloride (Selectol®), 3-[3-acetyl-4-(3-*tert*-butylamino-2-hydroxypropoxy)phenyl]-1,1-diethylurea hydrochloride (Fig 1), is a β_1-selective adrenergic antagonist with weak vaso- and bronchodilating effects Celiprolol was first introduced in 1985 and has been used as a racemate. Several pharmacokinetic studies [1–7] have been performed with the racemic drug In healthy human volunteers, the maximum plasma concentrations

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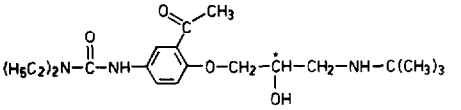

Fig 1 Structure of celiprolol The asterisk indicates an asymmetric centre

ranged from 114 to 662 ng/ml after therapeutic oral doses of 200 mg, and the terminal half-lives varied between 3 and 13.6 h Between 3 and 22% of the given racemic celiprolol dose was found unchanged in the urine. Metabolic clearance was described as being very low [2,5,7] or entirely absent [1,4]. The oral bioavailability was calculated to be between 30 and 70% [4,5,7], depending on the given dose and food intake

Radioligand binding studies showed that the enantiomers of celiprolol have different pharmacodynamic properties [8] and, therefore, enantiospecific evaluation of the pharmacokinetics is necessary. However, the available analytical methods [9–13] permit quantification of the racemate only We have therefore developed a sensitive method for the simultaneous determination of celiprolol enantiomers in human plasma and urine without any derivatization. The method is based on the direct resolution of (*R*)- and (*S*)-celiprolol with a chiral stationary phase. The applicability of this method was tested with one healthy volunteer who had received a single oral dose of 200 mg of racemic celiprolol

EXPERIMENTAL

Chemicals

Racemic celiprolol hydrochloride and celiprolol hydrochloride enantiomers were kindly provided by Rorer (Bielefeld, F.R.G.), racemic celiprolol base by Chemie Linz (Linz, Austria) and (*R*)-(+)-propranolol (internal standard) by ICI-Pharma (Planckstadt, F.R.G.). Solvents of analytical-reagent grade and the buffer solution of pH 10.00 used for sample preparation (1 l contained 3 092 g of boric acid, 3.728 g of potassium chloride and 1.756 g of sodium hydroxide) were supplied by E. Merck (Darmstadt, F.R.G.). Diethylamine was purchased from Fluka (Buchs, Switzerland).

Equipment

The high-performance liquid chromatographic (HPLC) system consisted of a Knauer (Berlin, F.R.G.) 64 pump, a Rheodyne (Cotati, CA, U.S.A.) 7125 injection valve with a 100-μl sample loop, a Shimadzu (Dusseldorf, F.R.G.) RF-535 fluorescence spectrometer, and a Knauer TY recorder. The vacuum centrifuge used was a Speed Vac concentrator (Bachofer, Reutlingen, F.R.G.). The thin-layer chromatographic (TLC) plates were scanned with a KM 3 chromatogram spectrophotometer (Carl Zeiss, Oberkochen, F.R.G.) and a

Model 56 recorder (Perkin-Elmer, Uberlingen, F.R.G.). Solutions were applied on to TLC plates using a Linomat III (Camag, Muttenz, Switzerland)

Sample preparation

In a screw-capped glass centrifuge tube, 1 ml of heparinized plasma was extracted with 5 ml of dichloromethane–propan-2-ol (95 5, v/v) after addition of 100 μl of buffer solution (pH 10.00), resulting in a pH of 9. The buffer solution also contained 100 ng of the internal standard [*R*-(+)-propranolol] After shaking for 15 min and centrifugation at 2500 *g* for 20 min at 10°C, 4 ml of the organic layer were carefully removed and transferred into another centrifuge tube. The extraction was repeated with 4 ml of dichloromethane–propan-2-ol and the combined organic phases (8 ml) were evaporated to dryness in a vacuum centrifuge at 30°C. The residue was dissolved in 250 μl of mobile phase

The work-up of urine samples (1 ml) was identical except that 1 ml of buffer solution was used (resulting in a pH of 10 in the aqueous phase before the extraction step)

Chromatography

The stationary phase was cellulose tris-3,5-dimethylphenyl carbamate polymer coated on macroporous silica (10 μm) commercially prepacked in a 250 mm×4.6 mm I.D. column (Chiralcel OD from J.T. Baker, Gross-Gerau, F.R.G.). Further, a precolumn (50 mm×4.6 mm I.D.) containing the same material was used for plasma and urine samples. Resolution of the enantiomers was achieved with *n*-hexane–propan-2-ol–diethylamine (85 20 0.1, v/v) as the mobile phase at a flow-rate of 1 ml/min. The injection volume was 100 μl (loop filling) and the mean pressure was 2.3 MPa. A xenon lamp with two monochromators was used as the light source for fluorescence detection with the detection wavelengths set at 350 nm for excitation and 480 nm for emission. The first eluting enantiomer was (*S*)-(−)-celiprolol with a retention time of 12.7 min and *R*-(+)-celiprolol eluted after 17.0 min. The internal standard eluted after 9.5 min and was detected with the wavelengths set at 295 nm for excitation and 345 nm for emission.

Drug and metabolite interferences

Several drugs for antihypertensive therapy were tested for interference with the assay. The substances tested belonged to the following groups of drugs: (a) diuretics (hydrochlorothiazide, furosemide, amiloride and triamterene); (b) calcium channel blockers (verapamil and nifedipine); (c) angiotensin-converting enzyme inhibitor (captopril); (d) other β-adrenoceptor-blocking agents (atenolol, acebutolol, carteolol, labetalol, metoprolol, oxprenolol, penbutolol and pindolol); (e) other drugs (reserpine, clonidine, α-methyldopa and dihy-

dralazine) A 1-μg amount of each drug was added to 1 ml of plasma containing 100 ng of (*R,S*)-celiprolol as well as to celiprolol-free plasma

Whether or not celiprolol metabolites interfere with the assay was investigated by fractional collection of the two celiprolol enantiomers in the present system and subsequent analysis of the concentrated samples in distinct chromatographic systems. Plasma and urine samples from human healthy volunteers obtained at different times after oral administration of 200 mg of (*R,S*)-celiprolol were studied. The chromatographic systems used were as follows

(i) On a Waters Nova Pak Resolve C_{18} (4 μm) HPLC column (150 mm $\times$ 3.9 mm I D.) (Waters–Millipore, Eschborn, F.R.G.) several mobile phases were tested at a flow-rate of 1 ml/min (a) acetonitrile–water–triethylamine (25 75 0.01, v/v); (b) methanol–water–triethylamine (75 30 0.02, v/v); and (c) methanol–water–triethylamine (40 60 0 02, v/v)

(ii) Volumes of 50 μl of the concentrated fractions were applied on a precoated silica gel 60 TLC plate with fluorescence indicator F_{254} (20 cm $\times$ 20 cm; Merck) The plate was developed at room temperature in an unlined glass tank (Desaga) containing chloroform–methanol–ammonia (80 14 1, v/v). The spectrophotometer was operated in the fluorescence mode (arrangement· monochromator–sample). The light source was an ST 41 mercury lamp with 365 nm as excitation wavelength. The emission was filtered with an FL 43 filter (slit 1 $\times$ 6 mm).

Short- and long-term stability

The short-term stability of (*R*)- and (*S*)-celiprolol in plasma and urine was tested after storing samples for five days at 8 °C For long-term stability studies, samples were stored for ten months at -22 °C.

Accuracy and reproducibility

Reproducibility experiments were performed at three different concentrations (5, 25 and 50 ng/ml enantiomer in plasma and 50 and 500 ng/ml enantiomer in urine) with ten samples for each concentration Intra-day coefficients of variation (C.V.) were calculated. Inter-assay variability was determined over a period of four weeks by analysing calibration graphs in the range 5–250 ng for plasma and 5–500 ng for urine. The accuracy was tested by a comparison of the concentrations in samples ($n = 10$, plasma and urine from a healthy volunteer) obtained applying the racemic assay of Buskin et al. [9] and those determined with the present method.

Recovery from samples

In order to define the recovery, spiked plasma (5, 25 and 50 ng/ml enantiomer) or urine samples (50 and 500 ng/ml enantiomer) were analysed and the peak areas compared with those of non-extracted celiprolol base dissolved in the mobile phase.

Linearity studies

The linear behaviour of the calibration graph was tested with spiked plasma and urine samples in the range 2 ng/ml–1 μg/ml racemate Peak areas or, when an internal standard was used, the peak-area ratios were calculated and plotted against the concentrations of (*R*)- an (*S*)-celiprolol.

Application of the enantiospecific celiprolol assay after a 200-mg oral dose

In order to test the applicability this method for pharmacokinetic studies in patients, one healthy volunteer received an oral dose of 200 mg of racemic celiprolol. At timed intervals before and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 14, 24, 32 and 48 h after dosage, venous blood was taken and transferred into heparinized screw-capped glass centrifuge tubes and the plasma was separated by centrifugation at 600 *g* for 20 min immediately after collection and stored at −22°C until assayed. In addition, urine was fractionally collected over 48 h (0–2, 2–4, 4–6, 6–9, 9–12, 12–24 and 24–48 h)

RESULTS AND DISCUSSION

The application of the chiral stationary phase for enantiospecific determination allows the direct chromatographic separation of the enantiomers of celiprolol, a procedure that can be performed without derivatization and is also possible for extracts from biological material With respect to the present method, the most striking advantage is that the fluorescence measurement of the parent compound permits selective quantification without the addition of possible interfering impurities, such as by-products resulting from derivatization procedures. Fig. 2 shows a chromatogram of celiprolol enantiomers with internal standard after extraction from plasma from a healthy volunteer compared with extracted drug-free plasma. The separation factor, α, was 1.42 with a resolution factor, *R*, of 2 23. The elution order was (*R*)-(+)-propranolol ($k' = 2.80$), (*S*)-(−)-celiprolol ($k' = 4.08$) and (*R*)-(+)-celiprolol ($k' = 5\,80$). In order to protect the analytical column from contamination with co-extracted plasma or urine constituents, a precolumn was necessary (especially with plasma samples).

Extraction procedure and relative recovery

A single extraction step did not yield sufficient extraction rates and therefore a second extraction step was introduced. This procedure led to relative recoveries from plasma samples of more than 90% (Table I) In urine samples (Table II) the relative recovery decreased at concentrations higher than 500 ng/ml enantiomer, reflected by non-linearity of the calibration graph above this range This fact can be neglected when concentrations occurring in pharmacokinetic studies are low For higher concentrations reduced sample volumes (0.2 ml) were used for the assay.

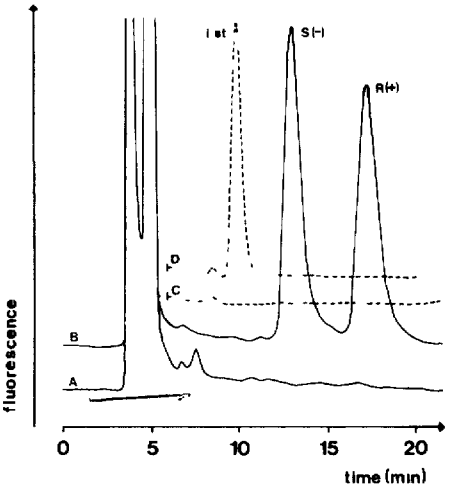

Fig 2 Chromatograms from plasma from a healthy human volunteer (A) Drug-free plasma, (B) = (*R*)-(+)- and (*S*)-(−)-celiprolol measured with excitation at 350 nm and emission at 480 nm, (C) and (D) same sample measured with excitation at 295 nm and emission at 345 nm in order to detect (*R*)-(+)-propranolol (internal standard, 1 st)

Interferences

There were no interferences from plasma constituents. Further, none of the tested antihypertensive drugs interfered with celiprolol Metabolites of celiprolol could not be detected.

Short- and long-term stability

In all instances the concentrations of both enantiomers of celiprolol remained constant throughout the observation periods

Linearity studies, accuracy and determination limit

Calibration graphs obtained for both enantiomers were linear over the range 5–500 ng, which covers clinically relevant concentrations The detection limit was 1 5 ng/ml enantiomer for plasma and 2 5 ng/ml enantiomer for urine at signal-to-noise ratios higher than 3. Intra- and inter-assay variabilities are summarised for plasma and urine in Tables I–IV Even without an internal standard the intra-day coefficients of variation were always below 9% for both enantiomers. When an internal standard was used the coefficients of variation improved to values $<5\%$ The racemate assay of Buskin et al [9] and the enantiospecific HPLC assay used here (sum of *R* and *S* enantiomers) gave congruent results at the tested sample concentrations. The linear equation obtained was $y=1.03x-0\ 17$; the correlation coefficient was 0 9999. In summary, the accuracy and the intra-assay and inter-assay variability indicate a high reliability.

TABLE I

INTRA-ASSAY ACCURACY AND PRECISION FOR DETERMINATION OF (*R,S*)-CELIPROLOL IN HUMAN PLASMA ($n=10$)

Compound	I S [a]	Concentration added (ng/ml)	Concentration found (mean ± S D) (ng/ml)	Coefficient of variation (%)	Recovery (%)
(*S*)-Celiprolol	No	50	50 1 ± 1 7	3 3	100
		25	25 6 ± 1 7	6 6	102
		5	4 7 ± 0 4	9 0	93
	Yes	25	24 7 ± 0 8	3 0	99
		5	5 5 ± 0 2	4 2	110
(*R*)-Celiprolol	No	50	46 9 ± 0 9	1 9	94
		25	23 9 ± 1 4	5 9	96
		5	4 7 ± 0 4	8 2	95
	Yes	25	24 0 ± 0 6	2 5	96
		5	5 3 ± 0 2	4 6	105

[a] (*R*)-(+)-Propranolol as internal standard (I S)

TABLE II

INTRA-ASSAY ACCURACY AND PRECISION FOR DETERMINATION OF (*R,S*)-CELIPROLOL IN HUMAN ($n=10$)

Compound	I S [a]	Concentration added (ng/ml)	Concentration found (mean ± S D) (ng/ml)	Coefficient of variation (%)	Recovery (%)
(*S*)-Celiprolol	No	500	415 8 ± 11 2	2 7	83
		50	48 0 ± 3 3	6 8	96
	Yes	500	490 0 ± 16 2	3 3	98
		50	51 0 ± 1 5	3 0	102
(*R*)-Celiprolol	No	500	428 5 ± 11 6	2 7	86
		50	51 2 ± 2 7	5 3	102
	Yes	500	505 0 ± 16 2	3 2	101
		50	52 5 ± 1 4	2 7	105

[a] (*R*)-(+)-Propranolol as internal standard (I S)

Preliminary pharmacokinetic study

The plasma concentration–time curves for (*S*)-(−)-celiprolol and (*R*)-(+)-celiprolol in one healthy volunteer are shown in Fig 3. The maximum plasma concentrations were 294 ng/ml (*R*)-(+)-celiprolol and 280 ng/ml (*S*)-(−)-celiprolol after a 200-mg oral dose. The $t_{1/2}$ values were 4.7 h for (*R*)-(+)-

TABLE III

INTER-ASSAY PRECISION FOR DETERMINATION OF (*R,S*)-CELIPROLOL IN HUMAN PLASMA ($n=6$)

Compound	I S [a]	Day[b]	Regression slope	Regression intercept (ng)	Correlation coefficient (r)
(*S*)-Celiprolol	No	1	0 10	0 12	0 9997
		2	0 06	−0 02	0 9998
	Yes	3	0 19	0 04	0 9992
		4	0 17	0 05	1 0000
(*R*)-Celiprolol	No	1	0 10	0 25	0 9995
		2	0 06	0 08	1 0000
	Yes	3	0 18	0 05	0 9990
		4	0 17	0 06	0 9990

[a] (*R*)-(+)-Propranolol as internal standard (I S)
[b] Over a period longer than four weeks

TABLE IV

INTER-ASSAY PRECISION FOR DETERMINATION OF (*R,S*)-CELIPROLOL IN HUMAN URINE ($n=6$)

Compound	I S [a]	Day[b]	Regression slope	Regression intercept (ng)	Correlation coefficient (r)
(*S*)-Celiprolol	No	1	1 28	0 55	0 9996
		2	1 57	0 67	1 0000
	Yes	3	0 30	0 02	0 9991
		4	0 46	0 19	0 9999
(*R*-Celiprolol	No	1	1 30	0 49	0 9994
		2	1 48	−0 31	1 0000
	Yes	3	0 33	0 02	0 9988
		4	0 51	0 67	0 9999

[a] (*R*)-(+)-Propranolol as internal standard (I S)
[b] Over a period longer than four weeks

celiprolol and 6 7 h for (*S*)-(−)-celiprolol. The cumulative renal excretion of (*S*)-(−)-celiprolol and (*R*)-(+)-celiprolol in the same volunteer is shown in Fig. 4. The amount excreted unchanged into urine was 14 06 mg (7.03% of the dose, *S/R* ratio 1 03). The unchanged renal clearance was 70.17 ml/min for (*S*)-(−)-celiprolol and 72 83 ml/min for (*R*)-(+)-celiprolol.

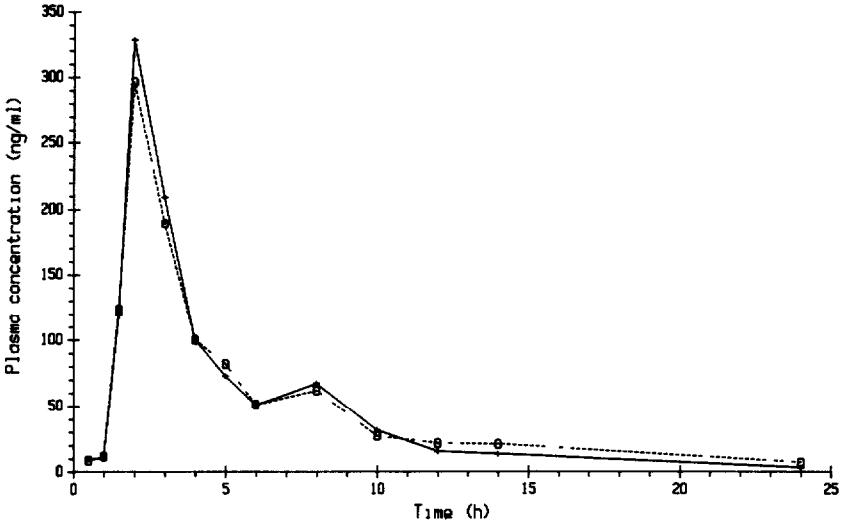

Fig 3 Plasma concentration–time curves for (+) (*R*)-(+)- and (○) (*S*)-(−)-celiprolol in one healthy human volunteer after oral administration of 200 mg of racemic celiprolol

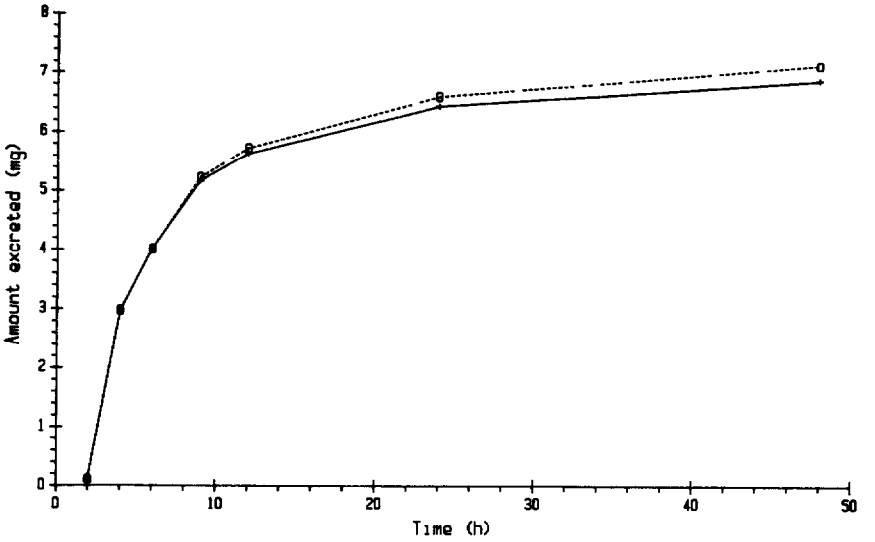

Fig 4 Cumulative renal excretion of (+) (*R*)-(+)- and (○) (*S*)-(−)-celiprolol in one healthy human volunteer after oral administration of 200 mg of racemic celiprolol

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