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Rapid Chiral Separation of Metoprolol in Plasma—Application to the Pharmacokinetics/Pharmacodynamics of Metoprolol Enantiomers in the Conscious Goat

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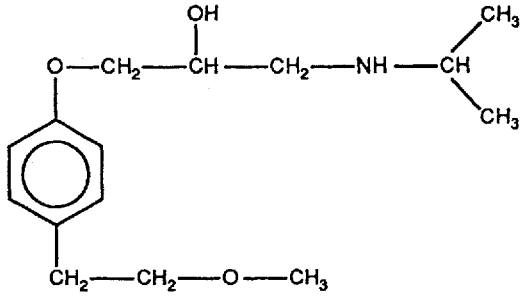
The plasma concentrations of metoprolol enantiomers have been determined by means of a direct phenyl carbamate-cellulose-based chiral high performance liquid chromatography assay using fluorimetric detection. This assay has been used to investigate the pharmacokinetics and pharmacodynamics of metoprolol enantiomers in the conscious goat. There is evidence that the pharmacokinetics of metoprolol in the goat occurs stereoselectively and that enantiomer–enantiomer pharmacokinetic interactions occur. *R*-Metoprolol is less effective in reducing the mean arterial blood pressure than *S*- and *R*/*S*-metoprolol

INTRODUCTION

For the routine analysis of enantiomers, the use of direct chiral separation methods involving high performance liquid chromatography (HPLC) is preferred. Using this approach, time-consuming and cumbersome derivatization reactions prior to chromatographic separation and detection can be avoided. Recently, a tris(3,5-dimethylphenyl carbamate)-cellulose chiral stationary phase has become commercially available which is especially suitable for the enantiospecific determination of beta-blocking drugs (e.g. Okamoto *et al.*, 1986, 1988; Krstulovic *et al.*, 1988; Straka *et al.*, 1988; Rutledge and Garrick, 1989; Aboul-Enein and Islam 1989; Ching *et al.*, 1989; Hartmann *et al.*, 1989; Straka *et al.*, 1990). Such enantiospecific bioassays are important tools for the study of the mechanisms of action of chiral drugs. At present, the significance of stereochemistry in both the pharmacodynamic action of drugs and their pharmacokinetic fate has become increasingly important (Drayer, 1988; Campbell, 1990).

In this paper an improved direct chiral separation method for metoprolol in plasma is described. This method has been evaluated by the investigation of both the system pharmacokinetic parameters and pharmacodynamics of *R*-, *S*- and *R*/*S*-metoprolol, as well as the relationships between the pharmacokinetics and pharmacodynamics of these compounds in the conscious goat.



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EXPERIMENTAL

Materials. *S*-Metoprolol oxalate was a gift from IBIS, The Netherlands. *R*/*S*-Metoprolol tartrate, *R*-metoprolol hydrochloride, 4-hydroxymetoprolol and *O*-demethylmetoprolol were obtained from Ciba-Geigy, Basle, Switzerland. The solutions for the infusion of the beta-adrenergic blocking drug (1 mg/kg) were prepared by dissolving adequate amounts of *S*-metoprolol oxalate, *R*/*S*-metoprolol tartrate or *R*-metoprolol hydrochloride in sterile 0.9% saline solutions, under aseptic conditions. *S*-Alprenolol was obtained from AB Hässle, Mölndal, Sweden. 2-Propanol, methanol, acetonitrile and *n*-hexane (Baker analysed HPLC reagent) were purchased from J. T. Baker Chemicals BV, Deventer, The Netherlands.

Instruments and chromatographic conditions. The HPLC apparatus consisted of the following components: pump model 510 (Waters, Etten-Leur, The Netherlands), a Schoeffel fluorescence detector, model FS 970 LL (Schoeffel Westwood, NJ, USA), an injector model 46K (Waters, Etten-Leur, The Netherlands) with a 2000 µL loop and an integrator model HP 3396A (Hewlett-Packard, Amstelveen, The Netherlands). The excitation wavelength was set at 225 nm, and a cut-off filter of 295 nm was used for the detection. The stainless steel column used was a Chiralcel OD (250 × 4.6 mm i.d.) from Daicel Industries (J. T. Baker Chemicals BV, Deventer, The Netherlands). The column temperature was ambient. A RCSS Silica guard-pak precolumn (Waters, Etten-Leur, The Netherlands) was used. The mobile phase consisted of a mixture of 2-propanol:*n*-hexane:diethylamine (25:75:0.05, v/v) with a flow of

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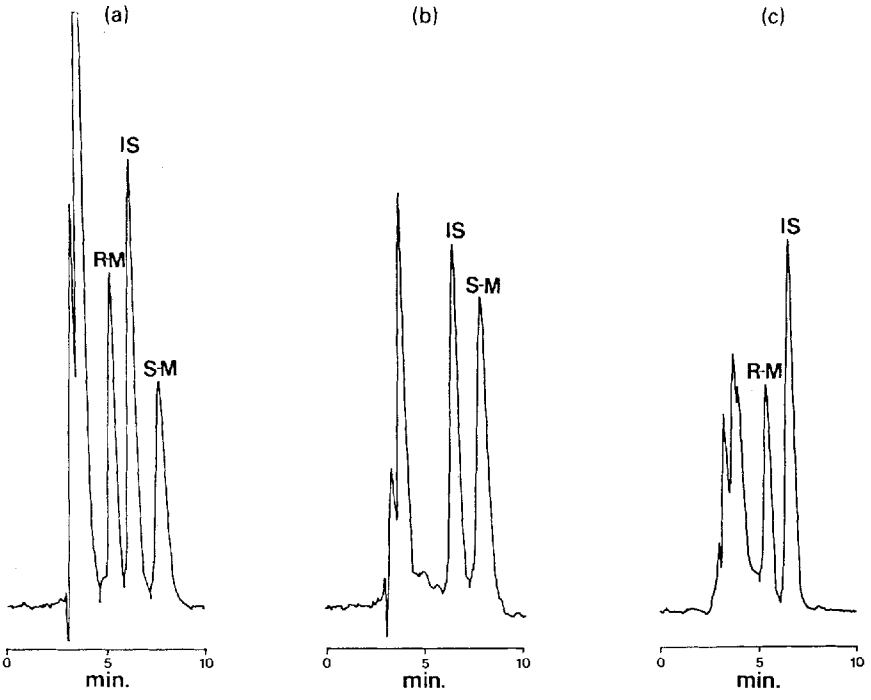
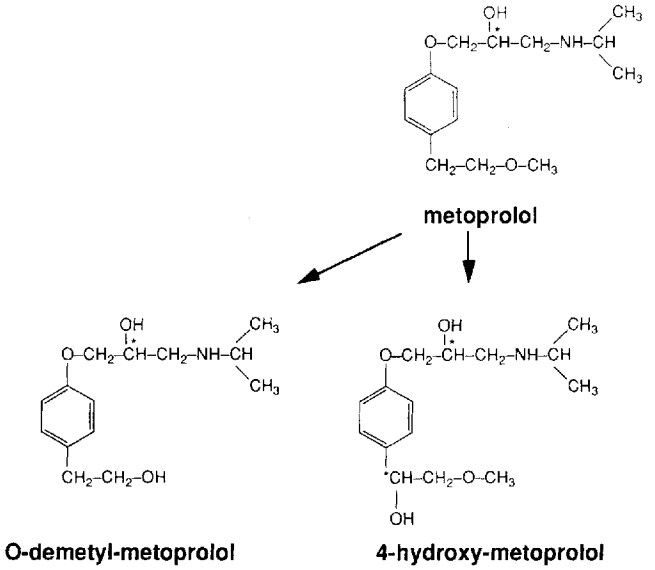


Figure 1. Representative HPLC chromatograms for the chiral separation of metoprolol enantiomers in plasma. (a) *n*-Hexane extract from 500 µL goat plasma at $t = 45$ min after the i.v. infusion of 2 mg/kg *R/S*-metoprolol. (b) *n*-Hexane extract from 500 µL goat plasma at $t = 45$ min after the i.v. infusion of 1 mg/kg *S*-metoprolol. (c) *n*-Hexane extract from 500 µL goat plasma at $t = 45$ min after the i.v. infusion of 1 mg/kg *R*-metoprolol.

1.0 mL/min. Prior to use, the mobile phase was filtered and degassed and the system permitted to reach an equilibrium for at least 12 h. The elution order of the metoprolol enantiomers was established by the injection of the respective enantiomers, which were available as pure reference compounds.

Sample preparation and assay validation. Plasma aliquots of 0.5–1.0 mL were mixed with 100 µL 1 N NaOH by vortexing for 10 s and mechanically shaken with 3 mL *n*-hexane for 10 min. After centrifugation at 2000 g for 10 min, the aqueous layer was frozen in a mixture of carbon dioxide and methanol and the upper *n*-hexane layer decanted into a clean centrifuge tube. The organic solvent was evaporated at 60 °C under a gentle stream of nitrogen and the residue reconstituted in 50 µL of a solution of the external standard (*S*-alprenolol, 2 µg/mL) in a mixture of *n*-hexane : 2-propanol (5 : 1, v/v) by vortexing for 15 s. The injection volume for the HPLC analysis was 35 µL. The extractions were performed in duplicate.

Every day of analysis, a calibration curve was prepared by adding known amounts of *R/S*-metoprolol to 0.5 mL drug-free plasma aliquots and performing the extraction procedure with *n*-hexane as described in the upper section. The peak heights of each enantiomer were divided by those from the internal standard and plotted versus the amount of enantiomer. Afterwards, linear regression analysis was performed. Quantitation occurred by an extrapolation of the calibration curve (3–150 ng/mL) prepared at the same day.



Scheme 1. Metabolic pathway for metoprolol. C* indicates the asymmetric carbon atom.

Animal studies. A goat (38 kg; $n = 2$) was anaesthetized with 30 mg/kg nembutal and 0.5 mg atropine by injection in the jugular vein. The trachea were intubated and the animal was respirated artificially in a closed system (oxygen : N_2O_2/ethrane (1:2); 50 mL/kg volume; 20 times per min). For the i.v. experiments, the carotid artery (aorta) as well as the jugular vein were cannulated with a polyethylene cannula (i.d. 2 mm, length 150 cm). Afterwards, the cannula was closed and left in a fixed position under the skin. 1–2 mL Albupen® 15% (Mycofarm, The Netherlands) was injected subcutaneously to prevent infection. After surgery, the animal was left undisturbed for at least one week. Prior to the infusion experiment, the unanaesthetized animal was weighed and then transferred to a special cage and held in an upright and fixed position. During the total experiment (up to 420 min), aorta blood pressure, ECG and heart rate were registrated. Blood samples (5–10 mL) were taken from the arterial canula (Monovette®, Sarstedt, Etten-Leur, The

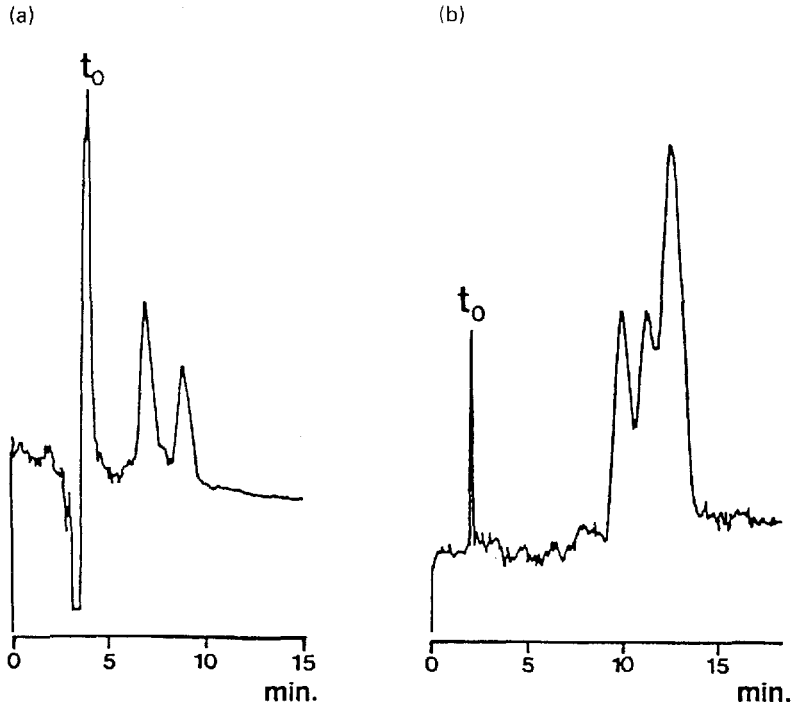


Figure 2. Chiral separation of metoprolol metabolites. (a) *O*-Demethylmetoprolol. Mobile phase: *n*-hexane : 2-propanol : diethylamine (75 : 25 : 0.05, v/v); flow 1 mL/min; 10 μg/mL solution in methanol, injection volume 25 μL. (b) 4-Hydroxymetoprolol. Mobile phase: *n*-hexane : ethanol : diethylamine (90 : 10 : 0.05, v/v); flow 1.2 mL/min; 20 μg/mL solution in methanol, injection volume 25 μL.

Netherlands). Blank blood samples were taken before the infusion.

For the i.v. experiments, *S*-metoprolol (1 mg/kg) *R*/*S*-metoprolol (1 and 2 mg/kg) or *R*-metoprolol (1 mg/kg) were administered by infusion into the venous cannula at $t = 0$ for 45 min. During the infusion, blood samples were drawn every 5 or 10 min. After the infusion, blood samples were collected over 15 or 30 min intervals up to 420 min. Plasma was separated by centrifugation for 15 min at 3000 g, transferred to a clean Monovette® tube and stored at −20 °C in the dark prior to analysis. In a few cases, urine was collected. At the end of the experiment, the cannula were closed and placed in a fixed position under the skin of the animal. The animal was left undisturbed for at least three days.

Pharmacokinetic and pharmacodynamic evaluation. Pharmacokinetics were calculated using a FarmFit®-program and interpreted according to the system dynamics approach (Van Rossum *et al.*, 1989). To account for the temporal delay between plasma concentrations and effects, the effect compartment approach was used (Sheiner *et al.*, 1979; Holford and Sheiner, 1982); this has been applied to beta-adrenoceptor drugs by several researchers (Reid and Meredith, 1990; Bortolotti *et al.*, 1989).

Table 1. Between-day variability of relative retention times and α-values of metoprolol enantiomers in plasma

Day	RT I.S.	RRT R − M	RRT S − M	α-value
1	7.22 ± 0.05	−1.56 ± 0.03	2.28 ± 0.05	2.76
3	7.03 ± 0.07	−1.47 ± 0.05	2.06 ± 0.06	2.71
7	6.95 ± 0.04	−1.46 ± 0.03	1.83 ± 0.07	2.63
10	6.65 ± 0.08	−1.27 ± 0.02	1.28 ± 0.05	2.37
14	6.25 ± 0.05	−1.02 ± 0.03	0.94 ± 0.02	2.10

RT = Retention time (min); RRT = relative retention time = RT (enantiomer) − RT (I.S.) (min); I.S. = internal standard *S*-alprenolol; R − M = *R*-isomer metoprolol; S − M = *S*-isomer metoprolol; α-value = K(S − M)/K(R − M); K = (RT (isomer) − T_0)/T_0.

RESULTS AND DISCUSSION

Assay validation

Typical chromatograms showing the excellent enantioseparation of *R*- and *S*-metoprolol in goat plasma using our developed direct chiral HPLC approach are given in Fig. 1. A baseline resolution, which enables the quantification of individual isomers was obtained. The *R*-enantiomer eluted before the *S*-enantiomer, which is in accordance with previous results (e.g. Straka *et al.*, 1990). The chromatographic run times permit the routine analysis of more than 40 samples containing metoprolol per day, which is approximately 1.5–2-fold more than reported by others (Ching *et al.*, 1989; Straka *et al.*, 1988; Takahashi *et al.*, 1988; Straka *et al.*, 1990). Other beta-blocking drugs, such as alprenolol and propranolol can also be analysed under the same conditions (Leloux and Maes, 1990a).

For the clean-up of goat plasma, a single-step extraction using *n*-hexane after alkalinization proved to be sufficient. No endogenous potentially interfering substances were found in the HPLC chromatogram, which implies that an additional acidic wash prior to the extraction, such as applied by Straka *et al.* (1990) was not necessary.

Scheme 1 shows the metabolism of metoprolol. Using our chiral separation method, the enantiomers of

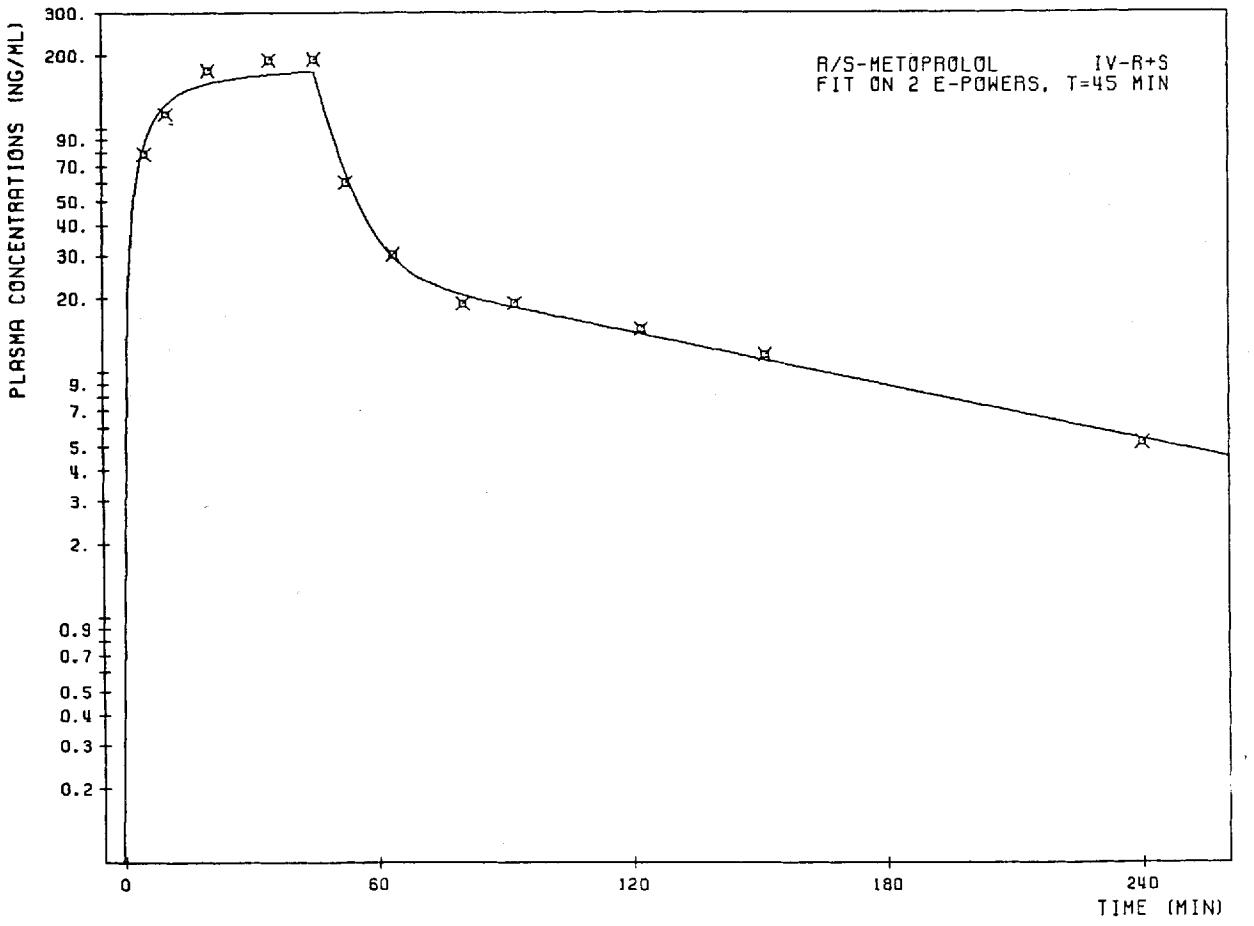


Figure 3. Plasma concentration–time curve for *R/S*-metoprolol after i.v. infusion of 1 ml/kg in the conscious goat.

Table 2. Pharmacokinetic system parameters of *R*-, *S*- and *R/S*-metoprolol after i.v. infusion in the conscious goat

Experiment	Cl	MRT	Vss	AUC
1.S – M	7.35	19.96	293.7	5169
1.S – M (1)	2.61	85.61	282.6	7268
1.R – M (1)	5.60	10.18	183.1	3392
1.S + R (1)	3.63	48.76	258.3	10480
2.S – M	4.27	26.69	199.5	8894
2.R – M	5.64	23.55	245.8	6732
2.S – M (1)	3.79	23.25	164.2	5007
2.R – M (1)	3.77	25.06	170.0	5036
2.S + R (1)	3.78	24.24	167.3	10050
2.S – M (2)	7.58	28.19	365.3	5012
2.R – M (2)	8.23	29.00	403.1	4620
2.R + S (2)	8.18	24.66	365.3	9292

AUC = area under the curve; Cl = clearance = D/AUC (L/min); MRT = mean residence time; Vss = apparent volume of distribution at steady state = D.TAUC/(AUC)^2 (L); S – M = *S*-metoprolol; R – M = *R*-metoprolol; R + S = racemic metoprolol; 1,2 = 1 resp. 2 mg/kg infusion.

Table 3. PK/PD values for metoprolol enantiomers in the conscious goat

Compound	Dose (mg/kg)	Goat	E_{max} (mm Hg)	k_{e_0} (L/min)	EC_{50} (ng//mL)
S-Metoprolol	1	2	– 12	0.00149	9
R-Metoprolol	1	2	– 8	0.0176	20
R/S-Metoprolol	1	2	– 22	0.0150	10
R/S-Metoprolol	2	2	– 29	0.00680	12
S-Metoprolol	1	1	– 23	0.00237	9

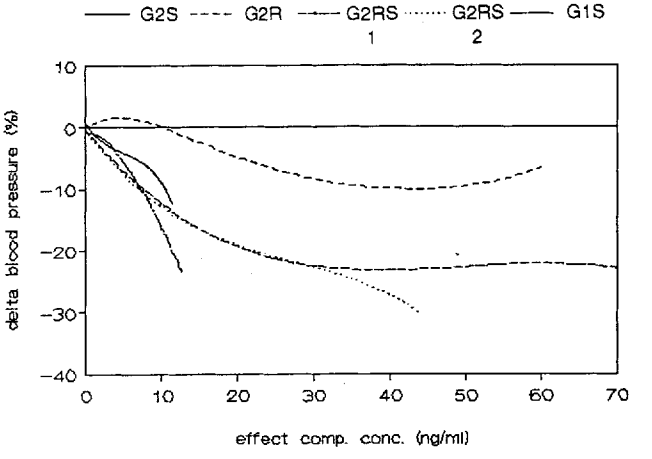


Figure 4. Concentration effect vs. compartment effect relationships of *R*-, *S*- and *R*, *S*-metoprolol (Spline function).

O-demethylmetoprolol have been separated; the elution order of the enantiomers is, however, uncertain. The four enantiomers of the diastereomeric molecule 4-hydroxymetoprolol were not separated using a chiral stationary phase (Fig. 2).

The recoveries after the extraction with *n*-hexane were 78.5 ± 10.4% for *R*-metoprolol and 78.3 ± 12.2% for *S*-metoprolol at 60 ng/500 µL plasma, and 72.6 ± 6.1% for *R*-metoprolol and 73.7 ± 4.9% for *S*-metoprolol at 150 ng/500 µL plasma, respectively (mean ± coefficient of variation; $n = 8$), indicating a non-stereoselective extraction. The ratio *S*-metoprolol/*R*-metoprolol in the racemic metoprolol reference compound was determined to range from

0.96 to 1.05 (mean = 1.00; $n = 8$). The assay limit of detection was 3 ng/mL plasma (signal-to-noise ratio = 3). The calibration curves were linear in the concentration range of interest (3–210 ng/mL) and the correlation coefficients ranged from 0.9671 to 0.9963 (mean = 0.9870; $n = 8$) for *R*-metoprolol and from 0.9808 to 0.9973 (mean = 0.9896; $n = 8$) for *S*-metoprolol, respectively. The within-day variability ranged from 3.28% to 9.82% (mean = 6.04%) and the between-day variability was approximately 6.0%. Our results are in close agreement with those presented by others (Straka *et al.*, 1990).

The coefficient of variation of the mean retention time and resolutions after the repeated injection of the racemic mixture ranged from 1.57 to 7.65%, thus indicating a good stability of the system. The retention times and α-values altered slightly between days (Table 1); it was therefore necessary to prepare a new calibration curve daily and to use the mobile phase for one week only. After flushing the column overnight with *n*-hexane at a low flow rate (0.4 mL/min) and reconditioning the column with a freshly prepared mobile phase for 2 h, the initial values for the retention times and α-values were re-evaluated. These findings suggest that the observations are caused by air humidity rather than by a deterioration of the stationary phase.

The coefficients of variation between duplicate measurements of metoprolol enantiomers extracted from goat plasma ranged from 0.1% to 16.6% (mean = 5.7%; $n = 63$) for *S*-metoprolol and from 0.6% to 18.7% (mean = 6.6%; $n = 39$) for *R*-metoprolol, respectively.

The extraction recoveries of *O*-demethylmetoprolol and 4-hydroxymetoprolol using *n*-hexane were lower than 10%. As a result, their presence in goat urine cannot be established using this enantiospecific assay and neither can they interfere with it. In conclusion, the assay validation was adequate for the routine application of this stereoselective bioassay.

Pharmacokinetics of *R*-, *S*- and *R*/*S*-metoprolol in the goat

The plasma *S*-metoprolol/*R*-metoprolol ratios ranged from 4 to 180 ng/mL for *S*-metoprolol and from 6 to 91 ng/mL for *R*-metoprolol in the first animal, and from 7 to 146 ng/mL for *S*-metoprolol and from 4 to 126 ng/mL for *R*-metoprolol in the second animal. In the i.v. infusion experiments, the plasma concentration–time curves were best described using a bi-exponential fit. Figure 3 shows a plasma concentration–time curve for a

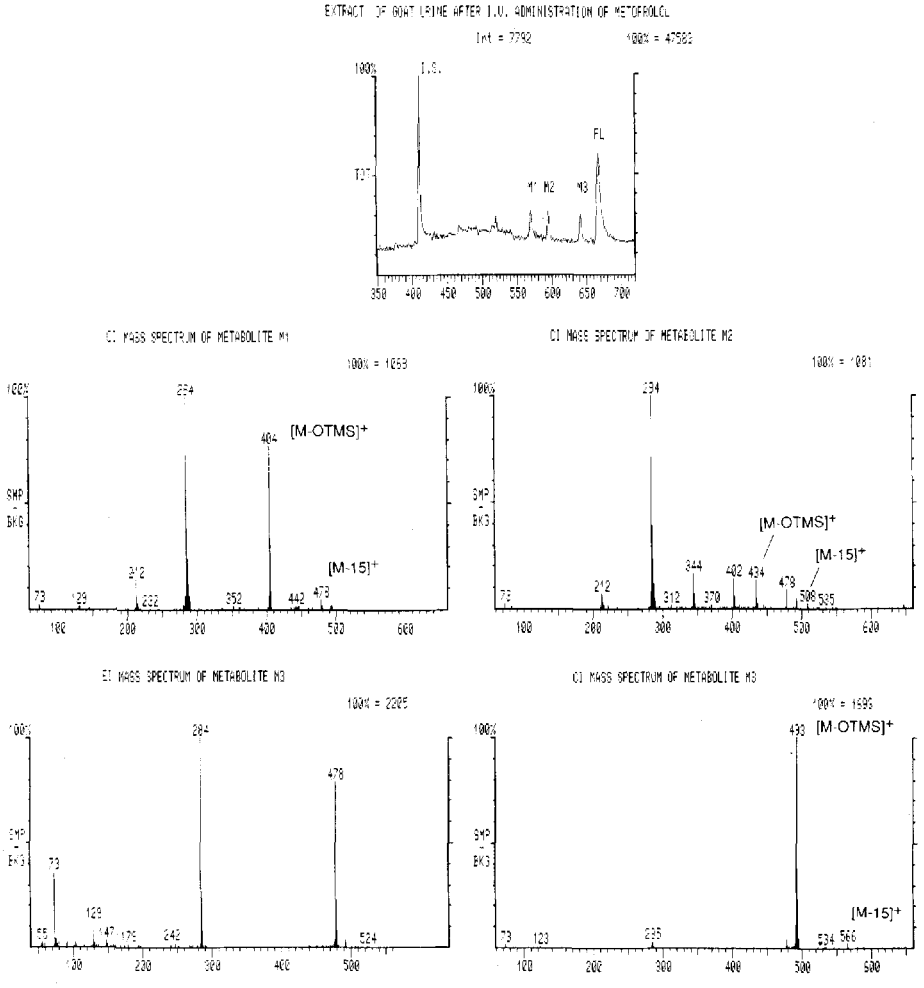


Figure 5. Total-ion chromatogram of urine extract from the goat, after the infusion of metoprolol. IS = internal standard bufuralol-*O*-TMS, M1 = *O*-demethylmetoprolol-*N*-TFA,bis-*O*-TMS (MW = 493), M2 = 4-hydroxymetoprolol-*N*-TFA,bis-*O*-TMS (MW = 523), M3 = unknown metabolite, Pl = Plasticizer di-2-ethylhexyl phthalate, released from the polypropylene vessel in which the urine has been stored prior to analysis.

goat after the i.v. infusion of 1 mg/kg *R*/*S*-metoprolol. Some pharmacokinetic system parameters after i.v. infusion of *S*-metoprolol, *R*-metoprolol, *R*/*S*-metoprolol (1 mg/kg) and *R*/*S*-metoprolol (2 mg/kg) in two animals are presented in Table 2. Since the cardiac output of the goat is not known, more detailed information cannot be obtained.

The evaluation of the pharmacokinetic parameters of metoprolol enantiomers indicates that the drug rapidly distributes in all tissues and that the body clearance occurs rapidly in the goat. In man, rat and dog similar observations have been reported (Borg *et al.*, 1975b; Regardh and Johnsson, 1980). Intriguingly, we additionally observed some differences in the pharmacokinetics of the enantiomers, as reflected in different AUC values and clearances, administered either alone or in combination with their optical antipode, suggesting that drug interactions occur between *R*- and *S*-metoprolol. Similar results have been obtained in *in vivo* experiments using goats, after the infusion of either metoprolol enantimers or the racemic mixture into the portal vein (unpublished results).

Pharmacokinetic/pharmacodynamic (PK/PD) modelling of metoprolol enantiomers

The results of our study clearly indicate that the *in vivo* pharmacodynamic action, i.e. the reduction of the mean arterial blood pressure, of *S*-metoprolol is larger than that of *R*-metoprolol, which is in accordance with findings presented by others (Walle *et al.*, 1988). The plasma concentration effect relationship of *R*-metoprolol in the goat strongly differs from those of its optical antipode and of the racemic mixture (Table 3; Fig. 4). Interestingly, the i.v. infusion of the relatively inactive *R*-isomer of metoprolol causes a considerable reduction of the mean arterial blood pressure in our animal experiment. This may be due by the formation of pharmacologically active metabolites, such as 4-hydroxymetoprolol (Borg *et al.*, 1975a, 1975b). According to Holford and Sheiner (1981), the presence of pharmacologically active metabolites may obscure the plasma concentration effect relationships of drugs. The presence of metabolites of metoprolol in plasma cannot be established using this chiral separation method (see section on 'Assay validation'), but traces of *O*-demethylmetoprolol, 4-hydroxymetoprolol and an unknown metabolite were found in goat urine (Fig. 5) by capilliary gas chromatography/mass spectrometry after solid-phase extraction and a trimethylsilylation–trifluoroacetylation derivatization reaction. Details of this procedure have been published elsewhere (Leloux and Maes, 1990b). Our results have revealed that these three metabolites were formed irrespective of the stereochemical constitution of the beta-blocking drug infused. The unknown metabolite may be a ring-hydroxylated derivative of *O*-demethylmetoprolol or its oxidation product. In goats, studies dealing with the metabolism of drugs (e.g. sulphonamides) have pointed out that hydroxylation and oxidation of xenobiotics might be considered as a major metabolic pathway (Kinabo and Nielsen, 1986; Nouws *et al.*, 1988).

CONCLUSION

A rapid and convenient improved direct enantioselective HPLC procedure for the routine determination of metoprolol enantiomers in plasma, based on a chiral tris(3,5-dimethylphenyl carbamate)-cellulose stationary phase has been developed. More than 40 samples can be analysed per day and the assay validation is adequate for routine analysis.

Furthermore, *in vivo* studies dealing with pharmacokinetic/pharmacodynamic modelling of metoprolol enantiomers in the goat have revealed that differences occur between the *in vivo* pharmacodynamic action of the less active *R*-isomer compared with that of the more active *S*-isomer and /or racemic mixture and that the pharmacokinetics of metoprolol in the goat occur stereoselectively.

It is evident that the development of reliable and accurate stereoselective bioassays for beta-blocking drugs may remarkably improve our knowledge with respect to the pharmacokinetics/pharmacodynamics of these drugs.

Acknowledgements

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