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## The 'free drug hypothesis' : fact or fiction?

Steeg, T.J. van

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# Chapter 5

## Mechanism-based PKPD modelling of $\beta$ -adrenoreceptor antagonists: role of plasma protein binding

TJ van Steeg<sup>1</sup>, VB Boralli<sup>2</sup>, EHJ Krekels<sup>1</sup>, P Slijkerman<sup>1</sup>, J Freijer<sup>3</sup>, M Danhof<sup>1</sup> and ECM de Lange<sup>1,4</sup>

<sup>1</sup>Division of Pharmacology, LACDR, Leiden University, Leiden, Leiden, The Netherlands

<sup>2</sup>Departamento de Analises Clinicas, Toxicologicas e Braomatologicas, Universidade de Sao Paulo, Sao Paulo, Brazil

<sup>3</sup>LAP&P Consultants BV, Leiden, The Netherlands

<sup>4</sup>Brains Online, Groningen, The Netherlands

## Summary

**Background and purpose** The goal of the current investigation was to determine the influence of plasma protein binding on *in vivo* pharmacodynamics of four  $\beta$ -blockers with a varying extent of plasma protein binding.

**Methods** Comparative PKPD studies were performed in conscious rats, using heart rate under isoprenaline-induced tachycardia as a pharmacodynamic endpoint. A previously proposed mechanism-based PD model was used to analyse the interaction between isoprenaline and the individual  $\beta$ -blockers, yielding estimates of the *in vivo* affinity ( $K_{B,vivo}$ ), which were compared to the *in vitro* affinity ( $K_{B,vitro}$ ).

**Results** The observed concentration versus heart rate profiles were described on basis of both total and free drug concentrations. For the total drug concentrations, the  $K_{B,vivo}$  estimates were 26, 13, 6.5, 0.89 nM for S(-)-atenolol, S(-)-propranolol, S(-)-metoprolol and timolol, respectively. The  $K_{B,vivo}$  estimates on basis of the free concentration were 25, 2.0, 5.2 and 0.56 nM for S(-)-atenolol, S(-)-propranolol, S(-)-metoprolol and timolol, respectively.

**Conclusions** For the selected  $\beta$ -blockers, the free plasma concentration appears to be the best predictor of drug response *in vivo*, since the *in vitro-in vivo* correlation for affinity on basis of free drug approximated the line of identity. In contrast, the slope of the *in vitro-in vivo* correlation on basis of total drug clearly deviated from the line of identity. Especially for the most highly bound drug S(-)-propranolol, the  $K_{B,vivo}$  on basis of total drug ( $13 \pm 4.7$  nM) deviated significantly from  $K_{B,vitro}$  ( $1.9 \pm 0.48$  nM).

### 5.1 Introduction

Binding to plasma proteins can have a major impact on a drug's pharmacokinetics (PK) and pharmacodynamics (PD) *in vivo*. At present the theoretical basis of the influence of (alterations in) plasma protein binding on PK is well-established. Specifically, depending on the extraction ratio, the clearance of a drug can be restrictive or non-restrictive with regard to plasma protein binding (Rowland and Tozer, 1995). With regard to the pharmacodynamics it is generally assumed that only the free drug concentration in plasma is responsible for the pharmacological effect of drugs *in vivo* ('free drug hypothesis'). Available experimental evidence suggests that for certain drugs (i.e. benzodiazepines, opiates, steroids) it may indeed be the free concentration that determines the intensity of the response (Cox *et al.*, 1998; Derendorf *et al.*, 1993; Mandema *et al.*, 1991). For other drugs, however, (i.e.  $A_1$  adenosine agonists) the opposite appears to be the case in the sense that it appears to be the total rather than the free concentration that determines the response (Van Der Graaf *et al.*, 1997). Thus the "free drug hypothesis" in pharmacodynamics is the subject to debate.

An important complication in examining the role of plasma protein binding in pharmacodynamics is that *in vivo* drug concentration-effect relationships are determined by multiple factors, related to the drug and the biological system. According to receptor theory, the potency of an agonist, as reflected in the  $EC_{50}$ , depends on both the receptor affinity of the agonist ( $K_A$ ) and its intrinsic efficacy ( $\varepsilon$ ). Moreover the  $EC_{50}$  may differ depending on the receptor density and the efficiency of the transduction in a given biological system (Black and Leff 1983; Kenakin 1993; Van der Graaf and Danhof, 1997). As a result the value of the  $EC_{50}$  of agonists is typically distinctly different from the affinity binding constant. This complicates the analysis of *in vitro* – *in vivo* correlations in pharmacodynamics. Recently, mechanism-based PKPD modelling concepts have been introduced, which are based on concepts from receptor theory, and in which a strict distinction is made between drug-specific and biological system-specific parameters to

characterise *in vivo* concentration-effect relationships (Zuideveld *et al.*, 2002, Van Der Graaf *et al.*, 1997). It has been shown that this modelling allows an independent estimation of the *in vivo* affinity constant  $K_A$  on the basis of plasma concentrations, which can be related to corresponding values obtained in *in vitro* bioassays. These approaches are uniquely suited to explore the validity of the free drug hypothesis.

The goal of the current investigation is to examine in a systematic manner the influence of plasma protein binding on *in vivo* pharmacodynamics. More specifically, the question is whether the total concentration or the free (unbound) concentration is the best predictor of drug effect *in vivo*. To this end the role of plasma protein binding on the pharmacodynamics of four  $\beta$ -blockers with a varying extent of plasma protein binding was determined in comparative PKPD studies in conscious rats. The  $\beta$ -blockers S(-)-atenolol, S(-)-metoprolol, S(-)-propranolol and timolol were selected as model drugs since they vary widely in plasma protein binding, ranging from almost no plasma protein binding for atenolol (~3%) to high plasma protein binding for propranolol (~95%), and furthermore differ widely in affinity for the  $\beta$ -adrenoceptor ( $\mu\text{M}$  –  $\text{nM}$ ) and in lipophilicity (Johnsson and Regardh, 1976; Mehvar and Brocks, 2001; Riddell *et al.*, 1987; Singh, 2005). Another advantage is the readily available PD endpoint (i.e. heart rate under isoprenaline-induced tachycardia) in both humans and animals (van Steeg *et al.*, 2007).

In the current study, mechanism-based PD modelling was used for the analysis of the interaction between isoprenaline and the individual  $\beta$ -blockers. The advantage of a mechanism-based approach is that an estimate of *in vivo* receptor affinity ( $K_{B,vivo}$ ) can be obtained for the  $\beta$ -blockers, which can be compared to the *in vitro* affinity ( $K_{B,vitro}$ ) from a functional assay (Van Steeg *et al.*, 2008). Comparison of the *in vitro*-*in vivo* correlation for receptor affinity on basis of free (unbound) drug and total drug will provide insight on whether the free or total drug is the main determinant for the pharmacological response for the  $\beta$ -blockers.

## 5.2 Methods

### *Animals*

All animal procedures were performed in accordance with Dutch laws on animal experimentation. The study protocol was approved by the Animal Ethics Committee of Leiden University (UDEC no. 02112 & 04078). Male Wistar Kyoto rats (301 g  $\pm$  37, n=71) obtained from Janvier (Le Genest Saint Isle, France) were housed individually at a constant temperature of 21°C and a 12-hour light/dark cycle. Prior to the surgery the rats were acclimatised for at least 5 days. The rats had ad libitum access to acidified water and food (laboratory chow, Hope Farms, Woerden, The Netherlands), except during the experimental procedures.

### *Surgery*

The rats were anaesthetised with a subcutaneous injection of 0.1 ml/100 g Ketanest-S® and an intramuscular injection of 0.01 ml/100 g Domitor®. During surgery the rats were placed on a heating pad to maintain body temperature at 37 °C. Seven days *prior* to the experiment, the rats were instrumented with four indwelling blood cannulas (Portex Limited, Hythe, Kent, England); two cannulas in the right jugular vein (Polythene 14 cm, ID 0.58 mm, OD 0.96 mm) for drug administration and one in the left and the right femoral artery (Polythene, 4 cm ID 0.28 mm, OD 0.61 mm + 20 cm ID 0.58 mm, OD 0.96 mm) for blood sampling and heart rate measurements respectively. The blood cannulas were subcutaneously tunneled and externalised at the dorsal base of the neck. To prevent blood clotting, the arterial cannulas were filled with a 25% (w/v) PVP solution in a 0.9 % saline solution containing 20 IU/ml heparin. The venous

cannula was filled with a saline solution containing 20 IU/ml heparin.

### *Experimental Design*

PKPD experiments were performed to characterise the PK and PD interaction between Isoprenaline and the  $\beta$ -blockers, S(-)-atenolol, S(-)-propranolol, S(-)-metoprolol and timolol.

*Isoprenaline* The PK and PD of Isoprenaline were determined and characterised as described previously (van Steeg *et al.*, 2007). In short, a population PK model was developed using data on the time course of isoprenaline. Thereafter the concentration-effect relationship was analysed using a mechanism-based PD model. The developed PK model was used to predict the concentrations of isoprenaline in the  $\beta$ -blocker interaction experiments, since a maximal effect is already observed at concentrations between the LOQ. In addition, sampling for both isoprenaline and the  $\beta$ -blocker would have required a too large volume of blood.

*Interaction of  $\beta$ -blockers and isoprenaline* Rats were randomly divided into nine groups. In two control groups the treatment consisted of 5 mg kg<sup>-1</sup> S(-)-atenolol without isoprenaline-induced tachycardia (non-isoprenaline (n=8)) and isoprenaline-induced tachycardia only (non-atenolol (n=9)). The rats in the seven treatment groups received S(-)-atenolol at doses of 0.5 (n=7), 1 (n=9) or 5 mg kg<sup>-1</sup> (n=9), S(-)-propranolol at doses of 1 (n=7) or 5 mg/kg (n=7), S(-)-metoprolol at a dose of 2.5 mg/kg (n=11) or timolol at a dose of 5 mg/kg (n=12) under isoprenaline-induced tachycardia. All  $\beta$ -blockers were dissolved in saline and administered as an intravenous infusion of 15 minutes (20  $\mu$ l min<sup>-1</sup>). Isoprenaline-induced tachycardia consisted of a continuous intravenous infusion of 5  $\mu$ g kg<sup>-1</sup> h<sup>-1</sup> isoprenaline in 0.1% SMBS saline solution. In the control groups, the infusions were replaced by vehicle solutions.

Serial arterial blood samples were collected in heparin coated tubes at pre-defined time intervals for determination of  $\beta$ -blocker concentrations. For all  $\beta$ -blockers blood samples of 100  $\mu$ l were taken and one sample was obtained before the start of the infusion (predose). For S(-)-atenolol a total of 14 blood samples were collected at 5, 10, 17.5, 20, 22.5, 32.5, 40, 70, 120, 180, 240, 360 and 480 min after the start of the infusion. For S(-)-propranolol a total of 14 samples were taken at 5, 10, 14, 15, 18, 20, 30, 36, 58, 66, 100, 134, 198, 240 and 360 min after the start of the infusion. For S(-)-metoprolol a total of 14 samples were obtained at 5, 10, 15, 17.5, 20, 25, 31, 36, 40, 45, 70, 90, 120 and 180 min after the start of the infusion. For timolol, a total of 13 samples were taken at 0, 5, 9, 15, 17.5, 20, 30, 45, 60, 90, 120, 180 and 240 min after the start of the infusion. Plasma samples were obtained by centrifugation (5 min; 5000 rpm) and stored at -20°C until analysis. The PD endpoint, heart rate, was recorded continuously throughout the experiment.

### *Pharmacodynamic measurements*

All experiments started between 8.00 and 9.00 AM to avoid influences of circadian rhythms. The baseline heart rate was recorded for 30 min; thereafter isoprenaline-induced tachycardia was recorded for 30 min before commencing with the  $\beta$ -blocker infusion. At the end of each experiment, approximately 480, 360, 180 and 360 min after the start of the infusion for S(-)-atenolol, S(-)-propranolol, S(-)-metoprolol and timolol, respectively, the continuous infusion of isoprenaline was stopped and heart rate was recorded for another 20 min. Arterial blood pressure and heart rate were measured from the cannulas in the femoral artery using a P10EZ-1 pressure transducer (Viggo-Spectramed BV, Bilthoven, The Netherlands), equipped with a plastic diaphragm dome (TA1017, Disposable Critiflo Dome, BD, Alphen a/d Rijn, The Netherlands). During the experiment the diaphragm dome was flushed with saline at a rate of 500  $\mu$ l h<sup>-1</sup> (Harvard 22-syringe pump, Harvard Apparatus Inc., South Natick, MA, USA). The pressure transducer was placed at

the level of the heart of the rats, when in normal position, and connected to a blood pressure amplifier (AP-641G, Nihon Kodhen Corporation., Tokyo, Japan). Heart rate was captured from the pressure signal. The signals were passed through a CED 1401*plus* interface (Cambridge, Electronic Design LTD, Cambridge, England) into a Pentium 4 computer using the data acquisition program Spike 2 (Spike 2 Software, version 3.11, Cambridge, England) and stored on a hard disk for off-line analysis.

#### *Protein binding*

For S(-)-propranolol protein binding was determined in WKY rat plasma, since this compound is considered to be highly protein bound. For S(-)-atenolol, S(-)-metoprolol and timolol values for protein binding were obtained from literature (Belpaire *et al.*, 1982; Rodgers *et al.*, 2005). These compounds are low and moderately bound in plasma and light alterations in plasma protein binding (i.e. due to strain) will, therefore, have less influence on the free fraction than for highly bound drugs.

#### *Drug analysis*

S(-)-atenolol, S(-)-metoprolol and S(-)-propranolol concentrations were quantified using HPLC with fluorescence detection. The HPLC-system consisted a LC-10AD HPLC pump (Shimadzu, 's Hertogenbosch, The Netherlands), a Waters 717 plus autosampler (Waters, Etten-Leur, The Netherlands), and a FP 920 fluorescence detector (Jasco Co, Tokyo, Japan). For S(-)-metoprolol the system was extended with a pulse damper (Antec Leyden, Zoeterwoude, The Netherlands). Fluorescence detection was performed using an excitation wavelength of 235 nm, 230 nm, 229 nm and an emission wavelength of 300 nm, 340 nm, 298 nm for atenolol, propranolol and metoprolol, respectively. Data acquisition and processing was performed using the Empower® data-acquisition software (Waters, Etten-Leur, The Netherlands).

Timolol concentrations were quantified using LCMS. The LC-MS system was running in SIM mode with electrospray (ESI) which had a positive polarity and sheath gas pressure of 35. Ion Sweep Gas Pressure and Aux Gas Pressure were both 10.0 and capillary temperature was set to 275 °C. Mass spectra ranged from 260 (width 1.0 for 0.2 s) to 317 (width 1.0 for 0.2 s).

*S(-)-atenolol* S(-)-atenolol concentrations were quantified using reversed phase HPLC following liquid-liquid extraction as described previously (van Steeg *et al.*, 2007). Chromatography was performed on Spherisorb ODS-2 3  $\mu$ m column (4.6 mm I.D. x 100 mm) (Waters, Millford, MA, USA) equipped with a refill guard column (2 mm I.D. x 20 mm) (Upchurch Scientific, Oak Harbor, WA, USA) packed with pellicular C18 (particle size 20-40  $\mu$ m) (Alltech, Breda, The Netherlands). The mobile phase consisted of 77.5% (v/v) 0.1 M sodium acetate buffer (pH 3.4) containing 5 mM octane-sulfonic acid (OSA) and 22.5% (v/v) acetonitrile. Sample (50  $\mu$ l plasma), internal standard (50  $\mu$ l Sotalol 5  $\mu$ g ml<sup>-1</sup> in water), sodium hydroxide solution (3 M, 100  $\mu$ l), water (200  $\mu$ l) and ethyl acetate (5 ml) were mixed, shaken (5 min) and centrifuged (4000 rpm, 10 min). The organic layer was taken and evaporated to dryness. Subsequently the residue was reconstituted in 100  $\mu$ l mobile phase and 50  $\mu$ l was injected into the HPLC-system.

*S(-)-propranolol* S(-)-propranolol concentrations were quantified using reversed phase HPLC following liquid-liquid extraction as briefly described below. Chromatography was performed on a chiralcel OD-R column (4.6 mm I.D. x 250 mm) (Diacel chemical industries LTD, Breda, The Netherlands) equipped with a commercially available guard column. The mobile phase consisted of 50% (v/v) 0.5 M sodium perchlorate aqueous solution and 50% (v/v) acetonitrile. Sample (50  $\mu$ l plasma), internal standard (50  $\mu$ l

sotalol 2  $\mu\text{g ml}^{-1}$  in water), sodium hydroxide solution (3 M, 100  $\mu\text{l}$ ), water (250  $\mu\text{l}$ ) and ethyl acetate (5 ml) were mixed, shaken (3 min) and centrifuged (4000 rpm, 10 min). The organic layer was taken and evaporated to dryness under vacuum (37 °C). Subsequently the residue was reconstituted in 100  $\mu\text{l}$  mobile phase and 50  $\mu\text{l}$  was injected into the HPLC-system. Linear calibration curves were obtained in the range 5-1000 ng ml<sup>-1</sup> ( $r > 0.995$ ,  $n = 8$ ) and the limit of quantification for S(-)-propranolol and R(+)-propranolol were 10 and 5 ng ml<sup>-1</sup>, respectively.

*S(-)-metoprolol* A validated normal phase HPLC assay was used for the quantification of S(-)-metoprolol enantiomers in rat plasma (Boralli *et al.*, 2005). Chromatography was performed using an amylose Tris (3,5-dimethylphenyl carbamate) coated on a 10  $\mu\text{m}$  silica gel substrate chiral column (Chiralpak AD, 250 mm  $\times$  4.6 mm). This column was equipped with a 4 mm  $\times$  4 mm Lichrospher 100 CN precolumn, 10  $\mu\text{m}$  particle size (Merck, Darmstadt, Germany) and was obtained from Daicel Chemical Industries (New York, USA). The mobile phase consisted of a mixture of hexane : ethanol: isopropanol : diethylamine (88:10.2:1.8:0.2, v/v/v/v) and the used flow rate was 1.2 mL/min. Sample (100  $\mu\text{l}$  plasma), sodium hydroxide aqueous solution (1M, 25  $\mu\text{l}$ ), sodium chloride (10 mg) and diisopropyl ether-dichloromethane (1:1, v/v, 4 mL) were mixed and centrifuged (1800  $\times$  g, 6 min). The organic layer was transferred to conical tubes and evaporated to dryness using a vacuum vortex evaporator (Buchler Instruments, Fort Lee, USA). The residues obtained were dissolved in 150  $\mu\text{L}$  of the mobile phase and 100  $\mu\text{L}$  was injected into the chromatographic system.

*Timolol* Timolol concentrations were quantified using LCMS as briefly described below. Chromatography was performed on a Zorbax Eclipse XDB-C8, 5  $\mu\text{m}$  (150  $\times$  4.6 mm) (Agilent technologies, California, USA). The mobile phase consisted of 60% (v/v) 0.1% formic acid buffer and 40% (v/v) acetonitrile with a flow rate of 400  $\mu\text{L min}^{-1}$ . Sample (50  $\mu\text{l}$  plasma), internal standard (50  $\mu\text{l}$  propranolol 500 ng/ml in water), sodium hydroxide solution (3 M, 100  $\mu\text{l}$ ), water (200  $\mu\text{l}$ ), heptane (2 ml) and ethyl acetate (3 ml) were mixed, shaken (3 min) and centrifuged (4000 rpm, 10 min). The organic layer was taken and evaporated to dryness under vacuum (37 °C). Subsequently the residue was reconstituted in 100  $\mu\text{l}$  mobile phase and 20  $\mu\text{l}$  was injected into the LCMS-system. Linear calibration curves were obtained in the range 1-1000 ng ml<sup>-1</sup> ( $r > 0.995$ ,  $n = 10$ ) and the limit of quantification for timolol was 1 ng ml<sup>-1</sup>, respectively.

#### *Data analysis*

The PK and the concentration-effect relationship of Isoprenaline served as an input for the data-analysis in this study and were characterised on basis of the operational model of agonism as described previously (van Steeg *et al.*, 2008). For the  $\beta$ -blockers, the PK and the PD interaction with isoprenaline were quantified using non-linear mixed-effects modelling as implemented in NONMEM software version V, level 1.1 (Beal and Sheiner, 1999). This approach takes into account structural effects and both intra- and interanimal variability. Parameters were estimated using the first-order conditional estimation method with  $\eta$ - $\epsilon$  interaction (FOCE interaction). Modelling was performed on an IBM-compatible computer (Pentium IV, 1500 MHz) running under Windows XP with the Fortran compiler Compaq Visual Fortran version 6.1. An in-house available S-PLUS 6.0 (Insightful Corp., Seattle, WA, USA) interface to NONMEM version V was used for data processing, management and graphical data display. Model selection was based on the objective function and the Akaike Information Criterion (AIC) (Akaike, 1974). Goodness-of-fit was determined by visual inspection of several diagnostic plots.

**Pharmacokinetics** The PK profiles of all  $\beta$ -blockers were most adequately described by fitting a standard three compartment model to the data. Interanimal variability of the PK parameters was described according to an exponential distribution model:

$$P_i = \theta \cdot \exp(\eta_i) \quad (1)$$

in which  $P_i$  is the individual value of model parameter  $P$ ,  $\theta$  is the typical value (population value) of parameter  $P$  and  $\eta_i$  is the random deviation of  $P_i$  from  $P$ . The values of  $\eta_i$  are assumed to be independently normally distributed with mean zero and variance  $\omega^2$ . Selection of an appropriate residual error model was based on inspection of goodness-of-fit plots. On this basis, a proportional error model was selected to describe residual error in the plasma drug concentration:

$$C_{obs,ij} = C_{pred,ij} \cdot (1 + \varepsilon_{ij}) \quad (2)$$

in which  $C_{obs,ij}$  is the  $j$ th observed concentration in the  $i$ th individual,  $C_{pred,ij}$  is the predicted concentration, and  $\varepsilon_{ij}$  accounts for the residual deviation of the model predicted value from the observed value. The values for  $\varepsilon_{ij}$  are assumed to be independently normally distributed with mean zero and variance  $\sigma^2$ .

**Pharmacodynamics** The developed PK models for isoprenaline and the  $\beta$ -blockers served as an input for the PD modelling. A mechanism-based model was applied to the concentration-effect relationship of isoprenaline under the assumption that the maximal obtainable effect in the system equals the maximal effect of isoprenaline (Kenakin 1993, Van Steeg et al., 2008). An estimate for the *in vitro* affinity of isoprenaline was obtained from literature (Doggrell et al., 1998). The efficacy ( $\tau$ ) of isoprenaline was estimated using equation 3 and the concentration-effect data reported previously.

$$E = E_0 + \frac{E_{max} \cdot \tau \cdot [A]^n}{(K_A + [A])^n + \tau \cdot [A]^n} \quad (3)$$

where  $E$  is the effect of the drug at concentration  $[A]$ ,  $E_0$  is the baseline heart rate,  $E_{max}$  is the maximum drug effect,  $K_A$  and  $\tau$  are the affinity and efficacy of isoprenaline and  $n$  is the slope factor, which determines the steepness to the curve. An effect compartment was used to resolve the observed hysteresis between the plasma concentration and the effect of the  $\beta$ -blockers. The following differential equation can be used under the assumption that the effect site concentration equals the plasma concentration in equilibrium.

$$\frac{dC_e}{dt} = k_{eo} \cdot (C_p - C_e) \quad (4)$$

where  $C_p$  represents the plasma concentration,  $C_e$  represents the effect-site concentration and  $k_{eo}$  is the first order rate constant describing drug transport.

Subsequently a mechanism-based PD interaction model for isoprenaline and  $\beta$ -blockers was used to describe the heart rate response in rats. The data obtained in the treatment and control groups were analysed simultaneously using equation 5.



$$E = E_0 + \frac{E_{\max} \cdot (\tau \cdot [A])^n}{(K_A + [A] + ([B] \cdot \frac{K_A}{K_B}))^n + (\tau \cdot [A])^n} \quad (5)$$

In which  $E$  is the overall effect of the agonist at concentration  $[A]$  and the antagonist at the concentration  $[B]$ ,  $E_0$  is baseline heart rate,  $E_{\max}$  is the maximum effect of the agonist,  $K_A$  and  $\tau$  are the affinity and efficacy of the agonist,  $K_B$  is the receptor affinity of the antagonist and  $n$  is the slope factor.

At the start of the experiment, the observed baseline heart rate is under influence of the endogenous agonist adrenaline. As a consequence, the heart rate drops below the initial baseline upon administration of the  $\beta$ -blocker. This observation was implemented in the model in a rather descriptive manner, since actual concentration-effect data on adrenaline and its interaction with S(-)-propranolol, S(-)-metoprolol or timolol were not available. In this respect it was assumed that the baseline in the first 20 min (baseline) of the experiment is elevated as described according to equation 6.

$$E_0 = \text{Base} + DB \quad (6)$$

In which  $E_0$  is the observed baseline heart rate in equation 4,  $\text{Base}$  is the true baseline heart rate and  $DB$  is the factor describing the difference between the observed baseline and the true baseline heart rate. The estimated  $E_{\max}$  for Isoprenaline ( $E_{\max\text{-iso}}$ ) is also under influence of this factor and therefore  $E_{\max}$  was defined as follows (eq. 7):

$$E_{\max} = E_{\max\text{-iso}} + DB \quad (7)$$

Interanimal variability of the PD parameters was described according to an additive (eq. 8) or an exponential (eq. 9) distribution model:

$$P_i = \theta + \eta_i \quad (8)$$

$$P_i = \theta \cdot \exp(\eta_i) \quad (9)$$

in which  $P_i$  is the individual value of model parameter  $P$ ,  $\theta$  is the typical value (population value) of parameter  $P$  and  $\eta_i$  is the random deviation of  $P_i$  from  $P$ . The values of  $\eta_i$  are assumed to be independently normally distributed with mean zero and variance  $\omega^2$ .

On this basis of visual inspection, a additive error model was chosen to describe residual error in the drug effect:

$$C_{\text{obs},ij} = C_{\text{pred},ij} + \varepsilon_{ij} \quad (10)$$

in which  $C_{\text{obs},ij}$  is the  $j$ th observed concentration in the  $i$ th individual,  $C_{\text{pred},ij}$  is the predicted concentration, and  $\varepsilon_{ij}$  accounts for the residual deviation of the model predicted value from the observed value. The values for  $\varepsilon_{ij}$  are assumed to be independently normally distributed with mean zero and variance  $\sigma^2$ .

### Drugs and Chemicals

S(-)-Atenolol, S(-)-Propranolol, Timolol and (-)-isoprenaline hydrochloride (isoprenaline) were purchased from Sigma-Aldrich BV (Zwijndrecht, the Netherlands). S(-)-metoprolol was kindly donated by Astra Hässle AB (Mölndal, Sweden). Ketanest-S® ((S)-ketaminebase) was purchased from Parke-Davis (Hoofddorp, The Netherlands). Domitor® (medetomidine hydrochloride) was obtained from Pfizer (Capelle a/d IJssel, The Netherlands). Polyvinylpyrrolidone (PVP) was obtained from Brocacef (Maarsen, The Netherlands). Heparin (20 IU/ml) was obtained from the LUMC (Leiden University Medical Center) Pharmacy (Leiden, The Netherlands) and 0.9% (g/v) saline from B. Braun Melsungen AG. (Melsungen, Germany).

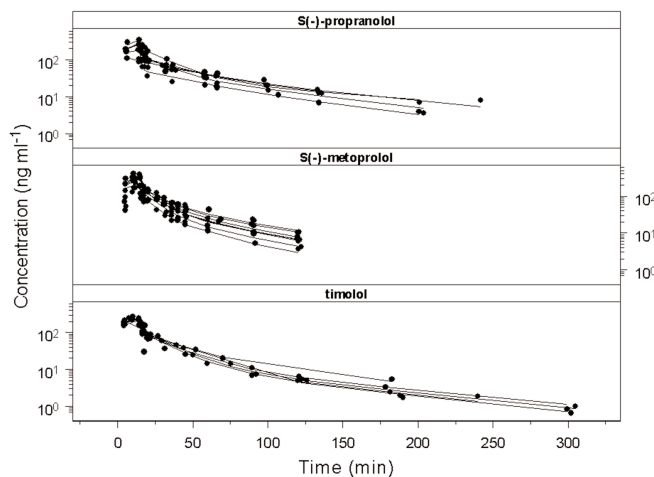


Figure 1, Concentration-time profiles for S(-)-propranolol, S(-)-metoprolol and timolol. The solid lines represent the individual model predictions and the dots represent the observed concentrations for the  $\beta$ -blockers under isoprenaline-induced tachycardia.

### 5.3 Results

**Pharmacokinetics** The concentration-time profiles for all  $\beta$ -blockers were described adequately by a three-compartment PK model. The PK model for S(-)-atenolol was reported previously and therefore not shown in the current publication (van Steeg *et al.*, 2008). The plasma PK profiles and the individual predictions for S(-)-propranolol, S(-)-metoprolol and timolol are shown in figure 1.

The PK parameter estimates for S(-)-atenolol, S(-)-metoprolol, S(-)-propranolol and timolol are displayed in Table 1. All structural PK parameters were estimated with good precision (CV<50%), except the first volume of distribution ( $V_1$ ) of timolol for which the coefficient of variation was 56%. The coefficients of variation for all other structural parameters ranged between 3.8 and 40% (Table I).

For all  $\beta$ -blockers, interanimal variability (IIV) for clearance ( $Cl$ ) was identified. For S(-)-atenolol interanimal variability was also observed for the second ( $V_2$ ) and third ( $V_3$ ) volume of distribution. For S(-)-propranolol, interanimal variability was also observed for the second volume of distribution ( $V_2$ ) and the intercompartmental clearance to the third compartment ( $Q_3$ ). Correlations between the values of interanimal variability were assessed by using a full omega matrix. A significant correlation was obtained for the interanimal variability on  $Cl$  and  $V_3$  for S(-)-atenolol. In addition a significant correlation was obtained between  $Cl$  and  $V_2$  for S(-)-propranolol. All random effects were estimated with good precision, except for the interanimal variability on  $Q_3$  for S(-)-propranolol (CV=66%) which was estimated with adequate precision.

Table 1, Population parameter estimates including coefficient of variation (CV%) for the PK models of S(-)-atenolol, S(-)-propranolol, S(-)-metoprolol and timolol

Parameter	Value (CV %)			
	S(-)-atenolol	S(-)-propranolol	S(-)-metoprolol	timolol
<b>Structural Parameters</b>				
Cl (ml min <sup>-1</sup> )	11.9 (3.8)	29.4 (10)	108 (5.7)	244 (7.9)
V1 (ml)	112 (8.3)	85.5 (25)	42.8 (40)	537 (56)
Q2 (ml min <sup>-1</sup> )	15.3 (8.0)	65.0(13)	41.7 (14)	373 (20)
V2 (ml)	171 (8.7)	540 (18)	1670 (12)	3340 (22)
Q3 (ml min <sup>-1</sup> )	8.73 (4.8)	12.0 (20)	228 (19)	55.9 (25)
V3 (ml)	857 (4.7)	930 (32)	1110 (11)	4770 (13)
<b>Interanimal Variability</b>				
$\omega_{Cl}^2$	0.036 (21)	0.094 (32)	0.034 (32)	0.057 (36)
$\omega_{V2}^2$	0.16 (30)	0.22 (48)	-	-
$\omega_{Q3}^2$	-	0.14 (66)	-	-
$\omega_{V3}^2$	0.023 (37)	-	-	-
$\omega_{Cl \rightarrow V2}^2$ (cov)	-	0.12 (30)	-	-
$\omega_{Cl \rightarrow V3}^2$ (cov)	0.025 (38)	-	-	-
<b>Residual error</b>				
$\sigma_{PK}^2$	0.027 (9.9)	0.033 (15)	0.076 (15)	0.040 (32)

**Pharmacodynamics** The individual PK parameter estimates served as an input for the PD analysis. The data for the PK and PD of isoprenaline have been analysed and reported previously (van Steeg *et al.*, 2007; van Steeg *et al.*, 2008). The earlier developed PK model for isoprenaline was used to simulate the concentrations of this agonist in the current  $\beta$ -blocker experiments. The concentration-effect relationship of isoprenaline was analysed with the operational model of agonism in order to obtain an estimate for the efficacy ( $\tau$ ).

The pharmacological effect on heart rate following isoprenaline and  $\beta$ -blocker administration was described using a previously proposed mechanism-based PD interaction model (Van Steeg *et al.*, 2008). All groups were analysed simultaneously and the fits for S(-)-atenolol, S(-)-propranolol, S(-)-metoprolol and timolol are shown in figure 2, 3 and 4, respectively. A steep increase in heart rate is observed directly after the start of the intravenous isoprenaline infusion. Subsequently, upon  $\beta$ -blocker administration, the heart

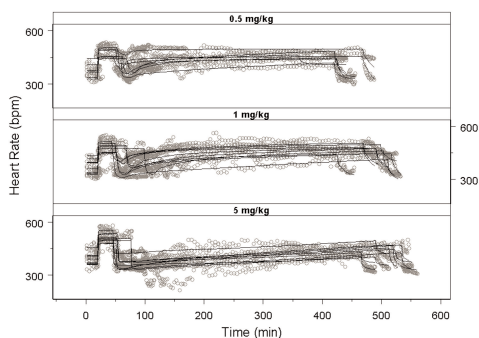


Figure 2, Plots per dose final PD model for S(-)-atenolol. The solid lines represents the individual model predictions and the open circles represent the heart rate observations for S(-)-atenolol under isoprenaline-induced tachycardia.

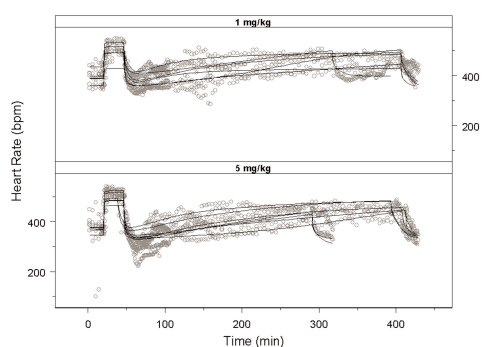


Figure 3, Plots per dose final PD model for S(-)-propranolol. The solid lines represents the individual model predictions and the open circles represent the heart rate observations for S(-)-propranolol under isoprenaline-induced tachycardia.

rate decreases again and slowly recovers in a concentration dependent manner as the antagonist is cleared from the system. Finally, after the stop of the steady state infusion of isoprenaline the heart rate decreases back to baseline (observed before drug administration).

Overall the mechanism-based interaction model described the PD profiles adequately and all parameters were estimated with good precision ( $1.8\% < CV < 37\%$ ). The parameter estimates for the simultaneous fit of all  $\beta$ -blockers under isoprenaline-induced tachycardia are displayed in table II.

The PD model was initially developed for S(-)-atenolol, since for this drug little plasma protein binding is observed *in vivo*. The PD model was modified to some extent for the current analysis.

Although not required for S(-)-atenolol, the use of an effect compartment for the description of hysteresis improved the fit significantly for the other (lipophilic)  $\beta$ -blockers ( $\Delta\text{MVOF} = -675$ ).

The use of an effect compartment model resolved hysteresis and the equilibration half-time between the central and the effect compartment ( $t_{1/2} = \ln 2/k_{eo}$ ) was 1.5, 10, 6.9 and 7.4 min for S(-)-atenolol, S(-)-propranolol, S(-)-metoprolol and timolol, respectively.

For a number of rats, the heart rate following  $\beta$ -blocker administration decreased below the initial baseline. Administration of S(-)-atenolol without the isoprenaline infusion resulted in a small decrease in heart rate and this effect was incorporated into the original baseline model by means of an sigmoid  $E_{\text{max}}$  equation (van Steeg *et al.*, 2008). The control group without isoprenaline induced tachycardia is essential for identification of the sigmoidal relationship. Since control profiles without isoprenaline-induced tachycardia were not obtained for S(-)-propranolol, S(-)-metoprolol and timolol the baseline model used in the current analysis needed adjustment. The drop below the initial baseline was described using the single parameter,  $DB$ , which represents the difference between the absolute baseline and the observed baseline

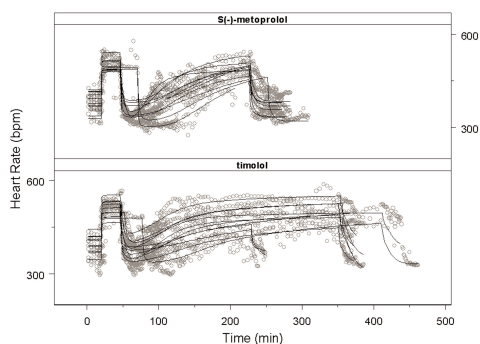


Figure 4, Plots final PD model for S(-)-metoprolol (2.5 mg/kg) and timolol (1 mg/kg). The solid lines represents the individual model predictions and the open circles represent the heart rate observations for S(-)-metoprolol or timolol under isoprenaline-induced tachycardia.

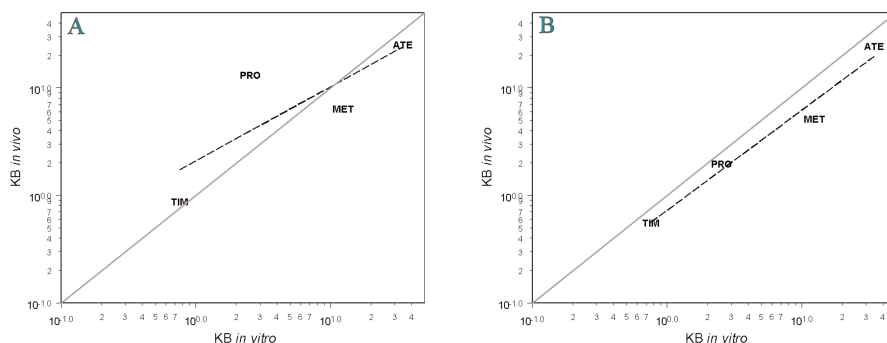


Figure 5, In vitro-in vivo correlation for the affinity of the  $\beta$ -blockers: S(-)-atenolol, S(-)-propranolol, S(-)-metoprolol and timolol. ATE, PRO, MET and TIM represent the estimates for  $K_B$  for the individual  $\beta$ -blockers. The solid and broken lines indicate the line of identity and the linear regression curve, respectively. A. The correlation on basis of total drug ( $r^2 = 0.70$ ). B. The correlation on basis of free drug ( $r^2 = 0.97$ ).

at the start of the experiment. This modification did not change the fit of S(-)-atenolol significantly, but improved the precision of the parameter estimates for all compounds.

In the course of the analysis, differences between the four  $\beta$ -blockers were investigated by defining parameters for each compound. The Hill coefficient for S(-)-metoprolol was found to be significantly different from one ( $1.6 \pm 0.18$ ). For the other  $\beta$ -blockers, the Hill coefficients were similar and not significantly different from one ( $n = 1.0 \pm 0.14$ ). Therefore in the final model, the Hill coefficient was assumed identical for S(-)-atenolol, S(-)-propranolol and timolol.

For all  $\beta$ -blockers, the *in vivo* affinity for the  $\beta$ -adrenoceptor ( $K_{B,vivo}$ ) was estimated using the interaction model. Initially the heart rate profiles were described using the total drug concentration *in vivo*. The  $K_{B,vivo}$  estimates were 26, 13, 6.5, 0.89 nM for S(-)-atenolol, S(-)-propranolol, S(-)-metoprolol and timolol,

Table 2, Population parameter estimates including coefficient of variation (CV%) for the mechanism-based PD interaction model. Simultaneous model fit of S(-)-atenolol, S(-)-propranolol, S(-)-metoprolol and timolol.

Parameter	Value (CV%)	IIV (%)
<i>Structural parameters</i>		
$E_0$ (bpm)	348 (1.8)	9.1
$E_{max}$ (bpm) <sup>a</sup>	130 (-)	20
$r^2$	247 (-)	-
$K_A = K_i$ (nM) <sup>b</sup>	30 (-)	-
$K_{B-ATE}$ (nM)	26 (21) 25 <sup>c</sup>	101
$K_{B-PRO}$ (nM)	13 (35) 2.0 <sup>c</sup>	119
$K_{B-MET}$ (nM)	6.5 (19) 5.2 <sup>c</sup>	70
$K_{B-TIM}$ (nM)	0.89 (25) 0.56 <sup>c</sup>	87
$n$	0.99 (14)	-
$n_{MET}$	1.7 (11)	-
$Ke0_{ATE}$ (min <sup>-1</sup> )	0.46 (37)	-
$Ke0_{PRO}$ (min <sup>-1</sup> )	0.066 (20)	-
$Ke0_{MET}$ (min <sup>-1</sup> )	0.10 (11)	-
$Ke0_{TIM}$ (min <sup>-1</sup> )	0.093 (24)	-
$D_B$ (bpm)	36 (20)	-
<i>Residual error</i>		
$\sigma_{PD-ATE}^2$ (bpm <sup>2</sup> )	1300 (28)	-
$\sigma_{PD-PRO}^2$ (bpm <sup>2</sup> )	1050 (20)	-
$\sigma_{PD-MET}^2$ (bpm <sup>2</sup> )	748 (18)	-
$\sigma_{PD-TIM}^2$ (bpm <sup>2</sup> )	1310 (13)	-

<sup>a</sup> Estimation from isoprenaline pharmacodynamics

<sup>b</sup> Obtained from literature

<sup>c</sup> Estimates KB on basis of free drug, CV% were equal to the values on basis of total drug

respectively (Table 2). Thereafter plasma protein binding was taken into account in the description of the PD. The affinity estimates on basis of the free concentration were 25, 2.0, 5.2 and 0.56 nM for S(-)-atenolol, S(-)-propranolol, S(-)-metoprolol and timolol, respectively. Finally, the  $K_{B,vivo}$  estimates on basis of the free and total concentration were compared to the *in vitro* affinities for the  $\beta$ -blockers ( $K_{B,vitro}$ ) obtained from literature (Table 3). For the total concentration, the relationship between the  $K_{B,vitro}$  and  $K_{B,vivo}$  crosses to the line of identity (Figure 5A). The slope of the line clearly deviates from the line of identity ( $K_{B,vitro} = K_{B,vivo}$ ). In addition, the  $K_{B,vivo}$  of S(-)-propranolol, the compound with the highest plasma protein binding, was significantly different from its  $K_{B,vitro}$ . The *in vitro-in vivo* correlation for  $K_B$  on basis of the free concentration is visibly more linear then on basis of total drug ( $r^2=0.97$  vs.  $r^2=0.70$ ). Although a parallel shift is observed compared to the line of identity for the correlation on basis of free drug ( $K_{B,vivo}$  below the line of identity for all compounds) the slope of the correlation appears to be identical to the line of identity (Figure 5B). The literature values of  $K_{B,vitro}$  for the  $\beta$ -blockers are displayed in table 3.

Table 3, Values for the *in vitro* affinity obtained from literature; the potencies of the  $\beta$ -blockers for were determined in rat right atria for all reported studies.

Compound	pA <sub>2</sub>	Slope	K <sub>B</sub> <sup>a</sup> (M)	Rat strain	Reference
Atenolol	7.16 ± 0.09	1.18 ± 0.10	6.90E-08 <sup>b</sup>	Wistar	Nandakumar 2005
Atenolol	7.3 ± 0.12	-	5.00E-08 <sup>b</sup>	Sprague-Dawley	Louis 1999
Atenolol	7.29 ± 0.13	1.02 ± 0.09	5.12E-08 <sup>b</sup>	Wistar (Normotensive)	Chiu 2004
Atenolol	6.93 ± 0.10	1.1 ± 0.07	1.17E-07 <sup>b</sup>	Sprague-Dawley	Juberg 1985
Metoprolol	7.60 ± 0.17	-	2.50E-08 <sup>b</sup>	Sprague-Dawley	Louis 1999
Metoprolol	7.26 ± 0.04	1.2 ± 0.07	5.49E-08 <sup>b</sup>	Sprague-Dawley	Juberg 1985
Propranolol	8.40 ± 0.32	-	3.90E-09 <sup>b</sup>	Sprague-Dawley	Louis 1999
Propranolol	8.29 ± 0.02	1.02 ± 0.06	5.10E-09 <sup>b</sup>	Wistar	Nandakumar 2005
Propranolol	8.56 ± 0.11	0.95 ± 0.07	2.75E-09 <sup>b</sup>	Wistar (Normotensive)	Chiu 2004
(-)-Propranolol	8.75 ± 0.04	1.2 ± 0.06	1.77E-09	Sprague-Dawley	Juberg 1985
Timolol	9.12 ± 0.05	1.2 ± 0.07	7.58E-10	Sprague-Dawley	Juberg 1985

<sup>a</sup> Calculated from the pA<sub>2</sub> value reported in the publications

<sup>b</sup> The actual affinity for the receptor is assumed to be half of the calculated concentration, since a racemate of *tw* was used in the studies only and the active enantiomer is considered responsible for the effects

## 5.4 Discussion and Conclusion

The aim of the current study was the determination of the influence of plasma protein binding on *in vivo* pharmacodynamics. More specifically, the question is whether the total concentration or the free (unbound) concentration is the best predictor of the effects of  $\beta$ -blockers on heart rate under isoprenaline-induced tachycardia. To address this question, comparative PKPD studies were performed in conscious rats for the  $\beta$ -blockers atenolol, metoprolol, propranolol and timolol. The free plasma concentration was found to be the best predictor of the  $\beta$ -blocker effects *in vivo*.

The pharmacological effect of a drug *in vivo* is the result of the distribution to and the interaction with its target (e.g. receptor or enzyme) (Rang *et al.*, 1999). Both target site distribution and target interaction are influenced by plasma protein binding (Herve *et al.*, 1994; Oie, 1986). The direct assessment of the free target site concentration *in vivo* is often impossible. The question whether the free or the total concentration is the main determinant of drug effect is, therefore, complex.

To determine the influence of plasma protein binding on the effects of drugs one should be able to distinguish between the contribution of plasma protein binding and other factors on the causal chain between drug administration and drug effect. In this study,  $\beta$ -blockers were chosen as their target site is the plasma compartment, leaving the influence of distribution as small as possible. Moreover for these drugs extensive knowledge is present on the plasma protein binding, the target interaction and the PD endpoint (heart rate).

The operational model of agonism was selected to describe the concentration-effect relationship of isoprenaline in order to obtain an estimate of efficacy for this agonist (Black and Leff 1983; Van der Graaf *et al.*, 1997). Subsequently an interaction model, which is based on the operational model of agonism, was selected to describe the overall heart rate effect in conscious rats following administration of isoprenaline and a single  $\beta$ -blocker (Van Steeg *et al.*, 2008). This mechanism-based modelling approach allowed the estimation of *in vivo* receptor affinity ( $K_{B,vivo}$ ) for the  $\beta$ -blockers.

The receptor affinity is a drug-specific property and is expected to be identical *in vitro* and *in vivo*. Estimation of  $K_{B,vivo}$  on basis of free and total drug plasma concentrations plasma and comparison with the estimates of  $K_{B,vitro}$ , thus, provided insight on whether the free or total drug concentration is the main determinant of pharmacodynamics *in vivo*.

The *in vitro-in vivo* correlation for affinity on basis of free drug concentration was linear ( $r^2 = 0.97$ ) and approximated the line of identity (Fig 5B). The slope of the *in vitro-in vivo* correlation on basis of total drug clearly deviated from the line of identity (Fig 5A). ). It was, therefore, concluded that the free drug concentration is the main determinant of the  $\beta$ -blocker effect on heart rate *in vivo*. Especially the  $K_{B,vivo}$  for the highly bound drug S(-)-propranolol on basis of total drug ( $13 \pm 4.7$  nM) deviated significantly from the  $K_{B,vitro}$  (mean value =  $1.9 \pm 0.48$  nM) (Juberg *et al.*, 1985; Louis *et al.*, 1999; Nandakumar *et al.*, 2005; Chiu *et al.*, 2004). Whereas on basis of free drug concentration the  $K_{B,vivo}$  ( $2.0 \pm 0.71$  nM) closely resembled the average  $K_{B,vitro}$ . The observed results are in agreement with a study reported by Yasuhara (Yasuhara *et al.*, 1985) in which the influence of alterations in plasma protein binding on pharmacodynamics was investigated, concluding that the free drug concentration determines the heart rate response for propranolol.

It was observed that for all  $\beta$ -blockers the  $K_{B,vivo}$  on basis of free drug was found to be higher than the  $K_{B,vitro}$ . Model misspecification with regard to the efficacy of isoprenaline ( $\tau$ ) may be the reason for this observation. The operational model of agonism was used to analyse the concentration-effect relationship of isoprenaline in which it was assumed that isoprenaline is the fullest agonist for the  $\beta$ -adrenoceptor system and also that the  $K_A$  is identical to the *in vitro* receptor affinity ( $K_i$ ). These assumptions, although considered valid, might result in a somewhat biased estimate for efficacy, which is used in the interaction model. In order to investigate this potential bias in the estimate for  $\tau$ , the observed heart rate profiles after administration of  $\beta$ -blockers were described on basis of the  $K_{B,vivo}$  (fixed to literature values). The model fit was not significantly different and  $t$  was estimated slightly larger ( $771 \pm 289$ ) than obtained from the concentration-effect relationship of isoprenaline ( $247 \pm 84$ ).

The equilibration half-time for S(-)-atenolol in the current analysis is 1.5 min. An equilibration half-time of 17 min was observed in a previous study (van Steeg *et al.*, 2007). In that study, the interaction with isoprenaline was not taken into account. It can, therefore, be concluded that the delay (hysteresis) between concentration and effect for S(-)-atenolol is caused by the interaction of the agonist with the antagonist at the receptor. Hocht *et al.*, (2006) studied the effect of metoprolol on heart rate in WKY rats and reported an equilibration half-time of 37 and 20 min for 3 and 10 mg kg<sup>-1</sup> metoprolol respectively (Hocht *et al.*, 2006). In the current study, a significant lower equilibration half-life (6.9 min) is observed for

2.5 mg kg<sup>-1</sup> S(-)-metoprolol. Like for S(-)-atenolol, the reduced hysteresis may to a certain extent be explained by the interaction with the (endogenous) agonist. However, for S(-)-metoprolol, S(-)-propranolol and timolol a significant hysteresis remained while taking into account the interaction with isoprenaline. Like for other drugs, lipophilicity seems to determine the magnitude of the hysteresis (Wierda and Proost, 1995). Thus, although heart rate is considered a direct effect and the  $\beta$ -blockers were selected as model compounds to minimise the distribution to the biophase, an effect compartment was needed to resolve hysteresis for the lipophilic  $\beta$ -blockers. Target site equilibration, receptor association/dissociation and transduction should therefore be considered as potential causes for the delay between the concentration in plasma and the eventual effect of the drug (Danhof *et al.*, 2007; Tuk *et al.*, 1997; Tuk *et al.*, 1998; Yassen *et al.*, 2005; Cleton *et al.*, 1999).

$\beta$ -adrenoceptors are located within the cardiovascular system and it is, therefore, unlikely that distribution of these compounds to the target site is the main reason for the observed hysteresis. For modelling purposes, the rate constants for receptor binding are assumed to be relatively high, such that binding instantly follows the change in plasma concentration. Contreras investigated the kinetics for binding at the  $\beta$ -adrenoceptors and reported the rate constants for association and dissociation for isoproterenol (isoprenaline), propranolol and timolol (Contreras *et al.*, 1986). A slow dissociation rate constant is reported for isoprenaline ( $k_{off} = 0.074 \text{ min}^{-1}$ ), however, this value was observed at 10°C. A direct comparison between the reported on- and off-rates and the hysteresis in this study is, therefore, not possible. The binding of the  $\beta$ -blockers to the receptor does not only depend on the association rate of these compounds, but also on the dissociation rate of isoprenaline. No other indications of slow binding kinetics have been reported in literature for isoprenaline and/or the  $\beta$ -blockers; the assumption of instant binding to the receptor needs further investigation.

For S(-)-metoprolol the  $K_{B,vivo}$  appears to be larger than the  $K_{B,vitro}$  for both the free and total drug assessment. In addition, the hill coefficient is significantly larger than one and different from the other three  $\beta$ -blockers. These observations may indicate the presence of an active metabolite for metoprolol. Alpha-hydroxy-metoprolol is one of the major metabolites of metoprolol and is formed upon oral administration in rats (Boralli *et al.*, 2005). This compound is known to possess  $\beta$ -adrenoceptor blocking activity, but a five times higher dose is needed to obtain the same effect as metoprolol (Regardh *et al.*, 1979). In dogs, approximately 5% of an intravenous dose of metoprolol was metabolised to alpha-hydroxy-metoprolol. It is, however, unknown to what extent this metabolite, or other active metabolites, are formed in rats after i.v. administration.

Although the heart rate profiles are described adequately by the PD model, the model slightly underpredicts the steep decrease in heart rate upon administration of S(-)-propranolol or S(-)-atenolol. For both compounds this underprediction is only observed in the highest dose group (5 mg kg<sup>-1</sup>) and is caused by rats (n=2) who experienced extremely low heart rate (bradycardia) upon  $\beta$ -blockers administration which might be due to other mechanisms (e.g. hypotensive effects) than the direct reduction in heart upon  $\beta$ -adrenoceptor binding.

In conclusion, the mechanism-based interaction model adequately described the effect on heart rate of the  $\beta$ -blockers S(-)-atenolol, S(-)-propranolol, S(-)-metoprolol and timolol under isoprenaline-induced tachycardia. It was seen that the effect of the  $\beta$ -blockers was determined by the free drug concentration and not by the total drug concentration, since on basis of the free drug concentration the *in vitro-in vivo* correlation for receptor affinity approximated the line of identity most.



## 5.5 Acknowledgements

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