Journal of Pharmacological and Toxicological Methods 59 (2009) 13–20

Contents lists available at ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Original article

Is urethane–chloralose anaesthesia appropriate for pharmacokinetic–pharmacodynamic assessment? Studies with carvedilol

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ARTICLE INFO

Article history:
Received 7 July 2008
Accepted 7 October 2008

Keywords:
Carvedilol
Pharmacokinetic–pharmacodynamic modeling
Urethane
Chloralose
L-NAME
Blood pressure
Heart rate

ABSTRACT

Introduction: The aim of the work was to establish the impact of urethane–chloralose anaesthesia on pharmacokinetic–pharmacodynamic (PK–PD) properties of carvedilol in control rats and L-NAME hypertensive animals. **Methods:** Male Wistar Rats were randomly divided into: control (n=12) with tap water to drink and L-NAME rats (n=12) with L-NAME solution (40 mg/kg/day) to drink for 2 weeks. Effects of carvedilol (1 mg kg^{-1}, i.v.) on blood pressure and heart rate were recorded during 3 h in conscious and urethane (500 mg kg^{-1}, i.p.) – chloralose (50 mg kg^{-1}, i.p.) anaesthetized rats. Carvedilol plasma pharmacokinetics was studied by means of traditional blood sampling. PK–PD modeling of carvedilol was made by means of an effect compartment model. **Results:** Neither urethane–chloralose nor L-NAME modified estimation of pharmacokinetic parameters of carvedilol. Although urethane–chloralose did not modify potency of carvedilol comparing with awake animals in control and hypertensive group, maximal negative chronotropic response was significantly greater in anaesthetized L-NAME rats in comparison to awake animals. Conversely, anaesthesia did not modify maximal chronotropic response to carvedilol in control rats. Whilst no differences were found in the estimated potency of carvedilol hypotensive response comparing control and L-NAME rats in both awake and anaesthetized conditions, maximal hypotensive effect of carvedilol was significantly greater in anaesthetized control and L-NAME animals in comparison to conscious rats. L-NAME rats showed a greater maximal hypotensive response comparing to control group. **Discussion:** Urethane–chloralose anaesthesia is an acceptable experimental condition for the evaluation of PK–PD properties of carvedilol, considering that it does not affect the potency of carvedilol for its chronotropic and hypotensive effect. Conclusions obtained from urethane–chloralose anaesthetized animals, regarding the impact of L-NAME treatment on PK–PD properties of carvedilol, did not differ from those obtained from conscious animals. Anaesthesia did not modify pharmacokinetic behaviour of carvedilol in both normotensive and L-NAME hypertensive rats.
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1. Introduction

Many pharmacological studies are difficult to perform in awake animals without exposing them to stress. To overcome this limitation, evaluation of pharmacokinetic and pharmacodynamic properties of drugs can be alternatively evaluated in laboratory animals under anaesthesia. In our laboratory, we have investigated pharmacokinetic–pharmacodynamic (PK–PD) properties of different antihypertensive drugs in animal models of hypertension using the microdialysis technique for continuous sampling of drug plasma levels (Bertera et al., 2007; Bertera, Mayer, Opezzo, Taira, & Höcht, 2008; Höcht, Di Verniero, Opezzo, & Taira, 2005; Höcht, Opezzo, & Taira, 2004a). Considering that microdialysis sampling in awake animals requires special equipment (Höcht, Opezzo, & Taira, 2004b), the experiments were performed in anaesthetized rats.

It is a well known fact that anaesthesia could modify animal physiology introducing artefacts in pharmacological studies (Claassen, 1994). Anaesthesia induced alterations also depend on the anaesthetic drugs used and their dosage. Among different anaesthetics used in pharmacological studies of antihypertensive drugs, mixture of urethane and chloralose is mostly employed because of its long duration of action (Claassen, 1994).

Several studies have described the effect of urethane–chloralose on animal physiology, including its effect on cardiovascular system (Le Noble, Struyker-Boudier, & Smits, 1987), drug metabolism (Loch, Potter, & Bachmann, 1995), and respiratory function (Claassen, 1994),

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doi:10.1016/j.vascn.2008.10.001

among others. Urethane–chloralose has shown to induce a decrease in basal blood pressure without exerting significant changes in heart rate (Le Noble et al., 1987). Urethane–chloralose also activates the sympathetic nervous system (Carruba, Bondiolotti, Picotti, Catteruccia, & Da Prada, 1987) and might change blood perfusion to different tissues, including the liver (Gumbleton, Nicholls, & Taylor, 1990). Moreover, different authors have described that the use of urethane at high doses could affect drug metabolism through an inhibition of cytochrome CYP3A, changing pharmacokinetic properties of some drugs (Loch et al., 1995; Meneguz, Fortuna, Lorenzini, & Volpe, 1999).

However, little is known regarding the effect of urethane–chloralose anaesthesia on the pharmacokinetic–pharmacodynamic behaviour of antihypertensive drugs in normotensive animals. It was found that urethane–chloralose mixture does not affect the biphasic pattern of blood pressure changes induced by clonidine (Bousquet, Gaertner, Feldman, & Bloch, 1977). In addition, it is important to stress out the lack of evidence of anaesthesia induced pharmacological alterations of antihypertensive drugs in animal models of hypertension.

Considering these antecedents, the aim of the present work was to establish the impact of the anaesthetic state induced by urethane–chloralose on the pharmacokinetic–pharmacodynamic properties of carvedilol in normotensive control rats and NG-nitro-L-arginine methyl ester (L-NAME) induced hypertensive animals.

Carvedilol was selected as a probe drug, considering that this beta-blocker is extensively used in the treatment of several cardiovascular disorders, including hypertension and heart failure (Carreira, Monteiro, Gon Alves, & Providência, 2006). Conversely to other beta-blockers, carvedilol also shows vasodilatory effects, and therefore theoretically this drug exerts a greater antihypertensive effect comparing with traditional beta-blockers (Sponer, Bartsch, Strein, Müller-Beckmann, & Böhm, 1987). Different lines of evidence also suggested that carvedilol is most effective than second generation beta-blockers, such as metoprolol, in the treatment of heart failure (Kohno et al., 2005). However, pharmacokinetic–pharmacodynamic properties of carvedilol were scarcely investigated in animal models of hypertension. PK/PD modeling of antihypertensive drugs in animal models of hypertension is a powerful tool in order to increase current knowledge of the mechanisms involved in the hypertensive state of experimental hypertension, allowing the identification of biomarkers and animal models for efficacy and toxicity, in order to predict antihypertensive response in different pathophysiological state of human hypertension (Höcht, Mayer, Opezzo, Bertera, & Taira, 2008).

2. Methods

2.1. Induction of hypertension

Male Wistar rats were used (220–250 g). Animal experiments were performed in accordance with the “Principles of laboratory animal care” (NIH publication No. 85-3, revised 1985).

Animals were maintained on a 12-h light/dark cycle and kept in a room at 22 ± 2 °C with the air adequately recycled. All animals were fed standard rodent diet (Asociación Cooperativas Argentinas, Buenos Aires, Argentina) with the following composition (w/w): 20% proteins, 3% fat, 2% fiber, 6% minerals, and 69% starch and vitamin supplements, containing the same amount of calories.

Rats were randomly divided into two groups: control ($n = 12$) with tap water to drink for 2 weeks and L-NAME hypertensive rats ($n = 12$) with L-NAME solution (40 mg/kg/day) to drink for 2 weeks.

2.2. Experimental design

In anaesthetized animal experiments, rats were anaesthetized with a mixture of chloralose (50 mg kg^{-1}, i.p.) and urethane (500 mg kg^{-1}, i.p.) dissolved in 5% glucose solution. A femoral vein was cannulated for the intravenous administration of isotonic solution containing carvedilol at a dose of 1 mg kg^{-1}. Left carotid artery was cannulated with a polyethylene cannulae and connected to a Spectramed P23XL pressure transducer (Spectramed, Oxnard, CA, USA) coupled to a Grass 79D polygraph (Grass Instrument, Quincy, MA, USA).

In conscious animal experiments, rats were anaesthetized with ether and the left carotid artery and left femoral vein were cannulated with polyethylene cannulae containing heparinized saline solution (25 U ml^{-1}). The cannulae were tunnelled under the skin and externalized at the back of the neck. Experiments were performed 24 h after cannulae placement in freely moving animals.

The day of the experiment, basal mean arterial pressure (MAP) and heart rate (HR) were estimated during an interval of 30 min. MAP was calculated as the sum of the diastolic pressure and one-third of the pulse pressure. HR was estimated tachographically by counting the pulsatile waves of arterial pressure recording.

Carvedilol was dissolved in saline solution containing equimolar concentration of beta-cyclodextrin and injected intravenously during 30 s. After carvedilol administration, MAP and HR were recorded every 5 min intervals and blood samples (70 µl) were collected from the arterial cannulae at the following time points: 5, 10, 15, 30, 60, 90, 120 and 180 min. The anaesthetic state was evaluated by the determination of the palpebral reflex and supplements of anaesthesia were administered if necessary. All experiments under anaesthetized conditions required for a supplementation of 5 mg chloralose and 50 mg urethane approximately 1 h after carvedilol administration.

2.3. Analytical determination of carvedilol in blood samples

Arterial blood samples (70 µl) were collected in 0.5 ml PCR tubes containing 5 µl of heparinized solution and gently mixed. Blood samples were centrifuged at 10,000 rpm for 10 min under controlled temperature (4 °C) to avoid either decomposition or biological activity. The plasma supernatant (20 µl) was carefully separated and deproteinized with acetonitrile (40 µl).

It is important to mention that blood sampling could alter pharmacokinetic and pharmacodynamic behaviour of antihypertensive drugs due to fluid loss (Höcht, Opezzo, Bramuglia, & Taira, 2006). Nevertheless, in our experimental protocol we only extracted approximately 560 µl of blood during 3 h period for estimation of plasma concentration of carvedilol. This volume is significantly lower than the recommended maximal volume of blood to be removed (3.5 ml) (Aimone, 2005), and therefore it could be suggested that blood loss during our experimental protocol did not affect PK–PD properties of carvedilol.

Levels of carvedilol in blood samples were measured by liquid chromatography with fluorescence detection using a Spherisorb ODS column 5 mm, C18, 250 × 4.6 mm (Waters Spherisorb, Wexford, Ireland) and a fluorescence detector (FL-3000, Thermo Finnigan, France). The excitation and emission wavelengths used were 238 and 350 nm, respectively. The optimal composition of the mobile phase was achieved by a mixture of distilled water, acetonitrile, triethanolamine (55:45:0.2), adjusted to pH 3.0 with phosphoric acid. Retention time of carvedilol in our chromatographic conditions was 6.4 ± 0.4 min. Coefficient of variation of the chromatographic method was less than 5% and the lower limit of quantification of carvedilol was 2.0 ng mL^{-1}. The intraday and interday coefficients of variation were 2.8 and 4.5, respectively. The method was linear in the range of 2–2000 ng ml^{-1}.

2.4. Analysis of the data

2.4.1. Pharmacokinetic analysis

Compartment analysis of carvedilol pharmacokinetics was used. The temporal profile of plasma carvedilol concentration following bolus dosing was described by a two-compartment, first-order elimination model. Non-linear least squares regression analysis was performed using the TOPFIT program (version 2.0, Dr. Karl Thomae Gmbh, Schering AG, Gödecke AG, Germany) that uses a cyclic three-stage optimization

routine (one-dimensional direct search; vectorial direct search/Hooke–Jeeves modified; Gauss–Newton/Marquadt modified). The area under the curve (AUC) of carvedilol levels vs. time (from 0 to infinity) was calculated using the trapezoidal rule. AUC_{0-180} was assessed by subtracting C_{180}/β from AUC0-∞, where C_{180} is the carvedilol concentration at 180 min after drug administration and β the terminal half-life. Clearance (Cl) and steady state volume of distribution (Vdss) were calculated by standard methods (Gibaldi & Perrier, 1982).

In addition, two-compartment model microconstants, k_{12}, k_{21} and k_{1e} were estimated using the TOPFIT program.

2.4.2. Pharmacokinetic-pharmacodynamic modeling of cardiovascular response to carvedilol

In the pharmacokinetic–pharmacodynamic (PK–PD) relationship study of carvedilol, plasma concentration of carvedilol and the data of blood pressure and heart rate change were used. Pharmacokinetic and pharmacodynamic data were fitted simultaneously for estimation of carvedilol PK–PD parameters. As a time delay between carvedilol plasma concentrations and their cardiovascular effects was observed, a PK–PD model with a separated effect compartment was used for analysis of the data. Previous studies by us and other authors found a good correlation between the cardiovascular effects of β-adrenoceptors blockers and their plasma levels by the application of PK–PD model with an effect compartment (Baek, Yun, Yun, & Kwon, 2008; Di Verniero et al., 2008; Höcht, Di Verniero, Opezzo, Bramuglia, & Taira, 2006; Höcht et al., 2005; van Steeg, Freijer, Danhof, de Lange, 2007).

The equation that describes the effect site concentration (Ce) of a two-compartment pharmacokinetic model is:

$$Ce = \left[\frac{k_{e0}*D}{V_1}\right]*\left[\left(\frac{k_{21}-\alpha}{(\beta-\alpha)*(k_{e0}-\alpha)}\right)*e^{-\alpha t}\right]+\left[\left(\frac{k_{21}-\beta}{(\alpha-\beta)*(k_{e0}-\beta)}\right)*e^{-\beta t}\right] + \left[\left(\frac{k_{21}-k_{e0}}{(\alpha-k_{e0})*(\beta-k_{e0})}\right)*e^{-k_{e0}t}\right]$$

where D is the dose, V_1 is the volume of distribution of the central compartment, α is the constant of distribution, β is the constant of elimination, k_{e0} is the equilibration rate constant, t is the time and k_{21} is the transfer microconstant from the peripheral to the central compartment.

A non-linear regression of these data was carried out using the ADAPT II software package (D'Argenio & Schumitzky, 1997) by means of the sigmoidal E_{max} equation:

$$Y = \frac{E_{max}*C_e(t)^{\gamma}}{EC_{50}+C_e(t)^{\gamma}}$$

where Y is blood pressure change expressed as ΔMAP (mmHg) or the chronotropic effect (expressed as % of basal HR), E_{max} is the maximal response, EC_{50} is the carvedilol concentration yielding half maximal response, γ the coefficient of Hill and $C_e(t)$ is the carvedilol concentration in the effect compartment at t time.

The following parameters of the PK–PD model were evaluated: EC_{50}, E_{max}, γ and $t_{1/2eq}$. The parameter $t_{1/2eq}$ is the equilibration half time between the plasma and the effect compartment and may be calculated from $\ln_2/K_{e0}$.

2.5. Statistical analysis

Normal distribution of the data and the variables of the study was verified using the Kolmogorov Smirnov test. Data were expressed as means±SEM. Statistical analysis was performed by two-way analysis of variance (ANOVA) and the test of Bonferroni as post-hoc test. Pharmacokinetic and PK–PD parameters were log transformed for statistical analysis in order to reduce heterogeneity of the variance.

Statistical tests were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Statistical significance was defined as $P<0.05$.

2.6. Drugs

The following drugs were used: carvedilol (Droguerías Saporiti, Buenos Aires, Argentina), urethane (Sigma, St Louis, MO, USA) and chloralose (United States Biochemical Corporation, Cleveland, Ohio, USA).

3. Results

3.1. General parameters

Basal values of MAP and HR of control and L-NAME rats under awake and anaesthetic conditions are shown in Table 1. No differences were found in body weight comparing control and L-NAME rats (data not shown). MAP was significantly higher in anaesthetized and awake L-NAME rats with regards to control animals (Table 1). Anaesthesia induced by urethane–chloralose significantly reduced MAP compared with awake animals. Neither anaesthesia nor L-NAME treatment modified HR in Wistar rats (Table 1).

3.2. Carvedilol pharmacokinetics

Fig. 1 shows the carvedilol concentration–time profile obtained from control (in A) and L-NAME rats (in B) under awake and anaesthetic conditions. A biexponential decay of plasma carvedilol levels was found in all experimental groups, compatible with a two-compartment pharmacokinetic model. Moreover, data fitted better to a two-compartment model (pooled AIC=63.3) with regard to a mono-compartment pharmacokinetic model (pooled AIC=73.4). The resulting pharmacokinetic parameters are shown in Table 2. No differences were found in the estimation of different pharmacokinetic parameters comparing anaesthetized and conscious animals in both normotensive and hypertensive rats. Hypertensive stage induced by L-NAME administration did not affect pharmacokinetic properties of carvedilol (Table 2).

In addition, no differences were found comparing microconstants of the two-compartment model obtained from all experimental groups (Table 3).

3.3. Pharmacokinetic–pharmacodynamic modeling of the chronotropic effect of carvedilol

The time course of HR change caused by i.v. administration of carvedilol (1 mg kg^{-1}) was studied in control (Fig. 2A) and L-NAME treated rats (Fig. 2B) under awake and anaesthetized conditions. Changes in HR were expressed as percentage of HR basal value (recorded during 30 min before administration of the drug). Anaesthetized animals showed a greater bradychardic response to carvedilol administration in L-NAME hypertensive animals (Fig. 2B) but not in normotensive rats (Fig. 2A). Although carvedilol induced a similar reduction in HR in awake L-NAME (19.4±2.3%, n=6) rats with regard to awake control animals (21.2±2.0%, n=6), anaesthesia increased responsiveness to carvedilol in L-NAME group (32.5±2.9%, n=6, $p<0.05$ vs. anaesthetized control rats) compared to normotensive rats (20.2±2.1%, n=6).

Table 1
Basal values of mean arterial pressure (MAP) and heart rate (HR) in experimental groups

	Control rats (n=12)		L-NAME rats (n=12)	
	Anaesthetized animals (n=6)	Awake animals (n=6)	Anaesthetized animals (n=6)	Awake animals (n=6)
MAP (mmHg)	93±8	115±3	139±6*	155±9#
HR (bpm)	411±6	441±13	449±15	426±12

*$p<0.05$ vs. Control anaesthetized rats.
#$p<0.05$ vs. Control awake rats.

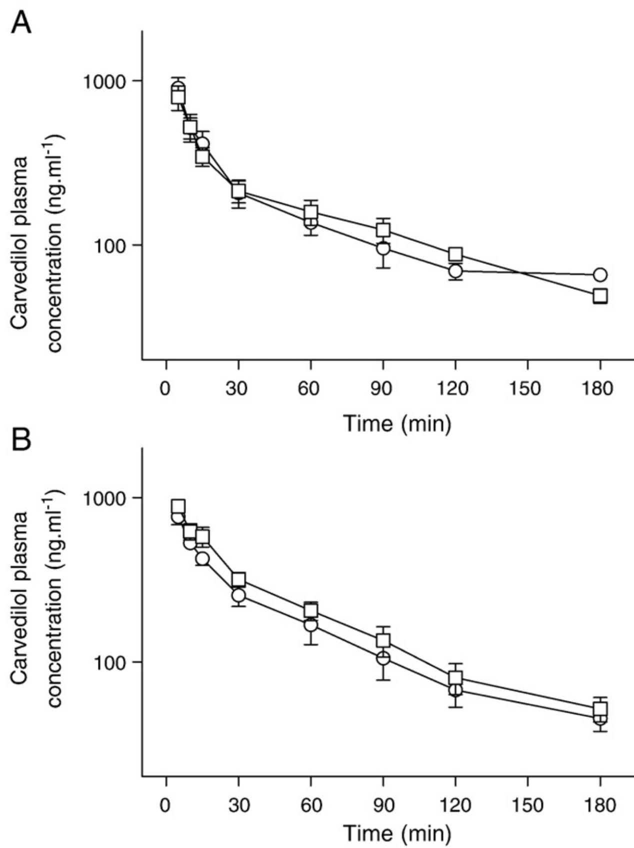

Fig. 1. Mean plasma concentration values of carvedilol vs. time in control normotensive rats (A) and L-NAME animals (B) under awake (circles) and anaesthized (squares) conditions after administration of 1 mg kg^{-1} of the drug. Each point shows the mean±SEM of six rats.

Table 4 shows PK–PD parameters obtained from PK–PD modeling of the chronotropic effect of carvedilol to an effect compartment model. Estimation of PK–PD parameters using the sigmoidal E_{max} equation requires the determination of the complete range of the pharmacological response after a single administration of the drug. Although in the present study we only show the results obtained after administration of 1 mg kg^{-1} of carvedilol, application of a higher dose of the drug (5 mg kg^{-1}) did not elicit a greater chronotropic effect (data not shown).

Table 2
Pharmacokinetic parameters of carvedilol obtained from arterial blood samples: AUC (area under the curve), α (constant of distribution), β (constant of elimination), Cl (clearance) and Vdss (steady state volume of distribution), C_0 (extrapolated maximal concentration) in control rats and L-NAME treated animals under awake and anaesthetized conditions after i.v. administration of drug (1 mg kg^{-1})

Pharmacokinetic parameter	Control rats (n=12)		L-NAME rats (n=12)	
	Awake (n=6)	Anaesthetized (n=6)	Awake (n=6)	Anaesthetized (n=6)
C_0 (μg ml^{-1})	1791±509	1628±298	1325±225	1511±126
α (h^{-1})	10.2±2.2	10.3±1.7	11.1±1.8	11.3±1.5
β (h^{-1})	0.70±0.13	0.64±0.11	0.73±0.06	0.64±0.08
Cl (ml min^{-1})	26.4±4.5	23.5±4.6	28.9±3.1	23.3±3.0
Vdss (l)	1.9±0.3	2.3±0.4	2.1±0.4	1.8±0.2
$AUC_{0-\infty}$ (ng ml^{-1} h^{-1})	703±87	828±130	609±65	792±116
AUC_{0-180} (ng ml^{-1} h^{-1})	676±37	739±73	548±60	732±108
Extrapolated area (%)	7.8±2.8	10.4±2.0	12.5±2.5	7.1±2.6

The data were expressed as mean±SEM of six animals.

Table 3
Microconstants estimation for a two-compartment model in control rats and L-NAME treated animals under awake and anaesthestized conditions after i.v. administration of drug (1 mg kg^{-1})

Microconstant	Control rats (n=12)		L-NAME rats (n=12)	
	Awake (n=6)	Anaesthetized (n=6)	Awake (n=6)	Anaesthetized (n=6)
k_{12} (h^{-1})	6.0±1.7	5.8±1.4	5.9±1.2	5.1±1.1
k_{21} (h^{-1})	2.0±0.7	3.5±0.8	4.6±1.3	4.4±1.4
k_{1e} (h^{-1})	2.3±0.7	2.2±0.4	1.8±0.4	2.3±0.4

The data were expressed as mean±SEM of six animals.

As shown in Table 4, effect compartment PK–PD model with sigmoidal E_{max} equation fitted well in all experimental groups, considering that mean Akaike criterion value did not differ comparing all groups and is compatible with the AIC value obtained in pharmacokinetic modeling. No differences were found in the estimated E_{max}, EC_{50}, $t_{1/2e}$ and γ parameters comparing control and L-NAME rats (Table 4). Although anaesthetic conditions induced by urethane–chloralose did not modify EC_{50}, $t_{1/2e}$ and γ parameters with regard to awake animals in control and hypertensive group, maximal chronotropic response was significantly greater in anaesthetized L-NAME rats with regard to awake hypertensive animals. Conversely, anaesthesia did not modify maximal chronotropic response to carvedilol in control normotensive rats (Table 4). Estimated PK–PD parameters for the negative chronotropic effect of carvedilol did not differ between L-NAME and control rats (Table 4).

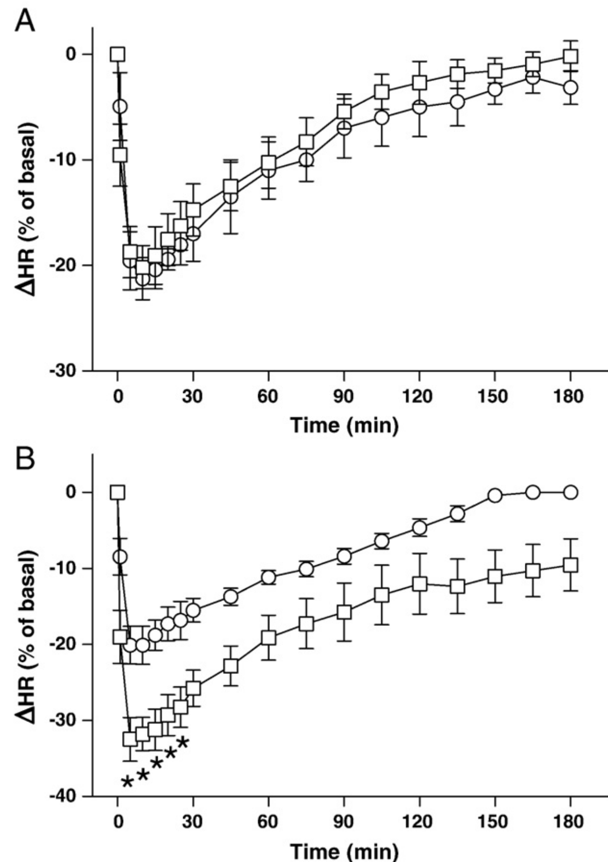

Fig. 2. Time course of heart rate changes (ΔHR, % of basal heart rate), after i.v. administration of carvedilol (1 mg kg^{-1}) in control normotensive rats (A) and L-NAME animals (B) under awake (circles) and anaesthized (squares) conditions after administration of 1 mg kg^{-1} of the drug. Each point shows the mean±SEM of six rats. *p<0,05 vs. awake animals.

Table 4
Resulting pharmacokinetic–pharmacodynamic parameters from the chronotropic effect of carvedilol in control rats and L-NAME treated animals under awake and anaesthetized conditions after i.v. administration of drug (1 mg kg^{-1})

Pharmacokinetic–pharmacodynamic parameter	Control rats (n=12)		L-NAME rats (n=12)	
	Awake (n=6)	Anaesthetized (n=6)	Awake (n=6)	Anaesthetized (n=6)
EC_{50} (ng ml^{-1})	168±24	228±31	166±29	221±37
E_{max} (%)	−24.8±1.8	−22.4±2.3	−22.8±2.2	−32.8±3.0[#]
γ	2.3±0.3	2.8±0.8	2.7±0.4	2.6±0.3
$t_{1/2\ eq}$ (min)	3.2±0.5	2.1±0.4	3.1±0.3	3.1±0.5
AIC	59.5	61.0	62.3	65.8

[#] $p<0.05$ vs. awake L-NAME rats.
The data were expressed as mean±SEM of six animals.
EC_{50}: concentration yielding half maximal response, E_{max}: maximal response, γ coefficient of Hill, $t_{1/2\ eq}$: equilibration half time between the plasma and the effect compartment, AIC: Akaike information criterion.

Table 5
Resulting pharmacokinetic–pharmacodynamic parameters from the hypotensive effect of carvedilol in control rats and L-NAME treated animals under awake and anaesthetized conditions after i.v. administration of drug (1 mg kg^{-1})

Pharmacokinetic–pharmacodynamic parameter	Control rats (n=12)		L-NAME rats (n=12)	
	Awake (n=6)	Anaesthetized (n=6)	Awake (n=6)	Anaesthetized (n=6)
EC_{50} (ng ml^{-1})	148±23	176±23	175±19	208±38
E_{max} (mmHg)	−27.4±1.8	−35.5±2.2[&]	−52.2±4.4*	−85.3±4.7*#
γ	1.7±0.4	2.3±0.4	1.6±0.4	2.1±0.4
$t_{1/2\ eq}$ (min)	7.2±2.5	4.3±1.1	6.8±2.3	3.8±0.7
AIC	63.3	62.5	80.1	88.5

*$p<0.05$ vs. control rats.
[#] $p<0.05$ vs. awake L-NAME rats.
[&] $p<0.05$ vs. awake control rats.
The data were expressed as mean±SEM of six animals.
EC_{50}: concentration yielding half maximal response, E_{max}: maximal response, γ: coefficient of Hill, $t_{1/2\ eq}$: equilibration half-life between the plasma and the effect compartment, AIC: Akaike information criterion.

3.4. Pharmacokinetic–pharmacodynamic modeling of the hypotensive effect of carvedilol

Fig. 3 shows the temporary course of MAP changes after carvedilol i.v. administration (1 mg kg^{-1}) in control (in A) and L-NAME (in B) treated rats under awake and anaesthetic conditions. Urethane–chloralose anaesthesia enhanced the hypotensive response to carvedilol in both control normotensive and L-NAME hypertensive animals. Nevertheless, in control group, Bonferroni post-test did not show any difference in the hypotensive effect of carvedilol in each single time point when comparing awake and anaesthetized rats. In addition, blood pressure lowering effect of carvedilol was increased in L-NAME rats with regard to control animals in both awake (control rats: ΔMAP: −23.7±1.5 mmHg, n=6; L-NAME rats ΔMAP: −43.5±5.3 mmHg, n=6, $p<0.05$) and anaesthetized conditions (control rats: ΔMAP: −35.6±4.6 mmHg, n=6; L-NAME rats ΔMAP: −84.2±5.4 mmHg, n=6, $p<0.05$).

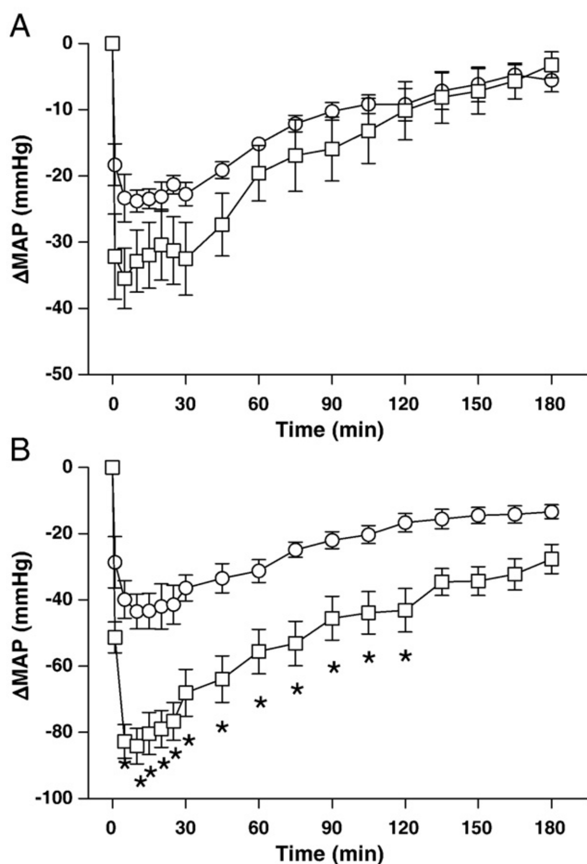

Fig. 3. Time course of changes in mean arterial pressure (ΔMAP, mmHg), after i.v. administration of carvedilol (1 mg kg^{-1}) in control normotensive rats (A) and L-NAME animals (B) under awake (circles) and anaesthized (squares) conditions after administration of 1 mg kg^{-1} of the drug. Each point shows the mean±SEM of six rats. *$p<0{,}05$ vs. awake animals.

Table 5 shows PK–PD parameters obtained from PK–PD modeling of the blood pressure lowering effect of carvedilol to an effect compartment model. As mentioned above, estimation of PK–PD parameters using the sigmoidal E_{max} equation requires the determination of the complete range of the pharmacological response after a single administration of the drug. Although in the present study we only showed the results obtained after administration of 1 mg kg^{-1} of carvedilol, application of a higher dose of the drug (5 mg kg^{-1}) did not elicit a greater hypotensive effect (data not shown).

As shown in Table 5, effect compartment PK–PD model with sigmoidal Emax equation fitted well in all experimental groups, considering that mean Akaike criterion value did not differ comparing all groups and is compatible with the AIC value obtained in pharmacokinetic modeling. No differences were found in the estimated EC_{50}, $t_{1/2e}$ and γ parameters comparing control and L-NAME rats (Table 5) in both awake and anaesthetized conditions. Maximal blood pressure lowering effect of carvedilol was significantly greater in anaesthetized control and L-NAME animals with regards to rats in awake condition. On the other hand, anaesthetized and awake L-NAME rats showed a greater hypotensive maximal response with regard to control group (Table 5). Anaesthetic conditions induced by urethane–chloralose did not modify PK–PD parameter estimation in both normotensive and L-NAME hypertensive rats (Table 5).

4. Discussion

Main results of our work show that urethane–chloralose anaesthesia does not modify pharmacokinetic properties of carvedilol in both normotensive and L-NAME hypertensive rats. Although anaesthetic condition induced by urethane–chloralose did not alter potency of carvedilol for its chronotropic and blood pressure lowering effect in both experimental groups, anaesthesia significantly enhanced maximal hypotensive response to carvedilol in control and L-NAME animals. Conversely, urethane–chloralose increased maximal bradychardic effect of carvedilol in L-NAME rats but not in normotensive rats. Finally, our results suggest a compromise of the sympathetic nervous system in the maintenance of the hypertensive stage in L-NAME rats, considering the greater efficacy of the hypotensive response to carvedilol in both awake and anaesthetized L-NAME rats with regards to control normotensive animals.

Although experiments in conscious animals are preferred in pharmacological science, this condition gives rise to some drawbacks. Particularly in the pharmacological evaluation of antihypertensive

drugs, measuring blood pressure by means of exteriorized catheters could be affected by the short-lived catheter patency (Kramer & Remie, 2005). As previously mentioned, use of sampling techniques, such as microdialysis, for PK–PD modeling of antihypertensive drugs in awake animals requires of special equipment and is also related to stress induction (Höcht, Opezzo, & Taira, 2004a,b). Considering these aspects, it is of interest to evaluate if anaesthesia modifies PK–PD properties of antihypertensive drugs during their evaluation in laboratory animals.

The impact of urethane–chloralose anaesthesia on pharmacokinetic–pharmacodynamic behaviour of carvedilol was evaluated in normotensive and L-NAME treated rats. Previously, we have successfully applied microdialysis sampling for PK–PD modeling of different antihypertensive drugs in anaesthetized animals. In the PK–PD study of carvedilol we failed to monitor plasma concentration of the beta-blocker by means of microdialysis because of high plasma protein binding of carvedilol (98%) (Frishman, 1998). It is important to mention that only the free drug fraction is available to dialyze through the microdialysis probe (Höcht, Opezzo, & Taira, 2004a,b). Therefore, in the present study we monitored total carvedilol plasma concentrations by traditional blood sampling. In addition, plasma protein of carvedilol could also be affected in all experimental groups by implantation of cannulae. It has been demonstrated that surgical implantation of cannulae in rats 24 h before the measurements induced an increment of alpha1-glycoprotein (Terao & Shen, 1983). Although alpha1-glycoprotein binds basic drugs, it has been demonstrated that carvedilol binds predominantly to serum albumin (Frishman, 1998). Therefore, it seems unlikely that increase in alpha1-glycoprotein due to cannulae implantation might affect carvedilol free fraction in our experimental conditions.

Oral administration of L-NAME induced a sustained increase in blood pressure after 2 days of drug administration (Biancardi, Bergamaschi, Lopes, & Campos, 2007; Gerová, 2000). MAP values reported for L-NAME hypertensive rats are similar to that reported in our study (Biancardi et al., 2007, Zicha, Dobesová, & Kunes, 2006). In accordance with other authors (Biancardi et al., 2007; Zicha et al., 2006), we also found that L-NAME does not change basal HR.

Urethane–chloralose anaesthesia may affect basal MAP and HR. Several authors have demonstrated that this anaesthetic regimen slightly reduces blood pressure without affecting HR (Le Noble et al., 1987; Smith & Hutchins, 1980). In our work, we found similar results regarding the effect of urethane–chloralose mixture on MAP and HR. Although anaesthesia significantly reduced MAP (approximately 10%) in normotensive and L-NAME hypertensive rats, it did not modify basal HR in both experimental groups.

Carvedilol is a lipophylic beta-blocker, whose most important pharmacokinetic properties are its high binding to plasma protein, its large volume of distribution and its hepatic biotransformation through different cytochrome P450 isoenzymes (CYP2D6 and CYP1A2) (Frishman, 1998; Ishida et al., 2008; Keating & Jarvis, 2003; Ruffolo, Boyle, Venuti, & Lukas, 1993). It is also important to mention that R- and S-enantiomers of carvedilol show similar clearance rates (83.8 vs. 96.1 ml min^{-1} kg^{-1}) (Fujimaki, 1992). In addition, it was also shown that carvedilol clearance is reduced in debrisoquinine slow metabolizers and patients under treatment with CYP2D6 inhibitors (Frishman, 1998).

Although some carvedilol metabolites retain β-adrenoceptor blocking activity also have weak vasodilator efficacy, the clinical importance of these properties are unknown (Frishman, 1998). Preclinical studies show that only 4'-hydroxycarvedilol (M4) appear to contribute to cardiovascular activity of carvedilol. Great interspecies differences were found in carvedilol metabolism (Schaefer et al., 1998). Although carvedilol is extensively metabolized in rats, mice and dogs, rats showed the simplest metabolite profile, considering that primary metabolites were formed by hydroxylation of the carbazolyl ring, with subsequent glucuronidation (Schaefer et al., 1998). More importantly, rats do not metabolize carvedilol to the active metabolite M4 (Schaefer et al., 1998). Carvedilol metabolism in rats also differs from biotransformation in human being, considering that *O*-Demethylation of carvedilol was observed in humans (Neugebauer & Neubert, 1991), but not in rats (Schaefer et al., 1998).

Urethane–chloralose may affect drug hepatic biotransformation. A single dose of urethane inhibits CYP 3A but increases CYP 2E1 and CYP 1A activity (Meneguz et al., 1999). Conversely, Loch et al. (1995) et al have shown that chloralose does not affect cytochrome P450 activity. In our study, urethane–chloralose anaesthesia did not modify carvedilol plasma levels in both normotensive and L-NAME treated animals and consequently estimated pharmacokinetic parameters were similar comparing rats under awake and anaesthetic conditions. Our results are in accordance with the fact that urethane–chloralose anaesthesia does not affect activity of CYP2D6 isoenzyme, the mayor isoenzyme involved in carvedilol clearance. Urethane dose used in the present work is lower than the dosing used by Loch et al. (1995) and Meneguz et al. (1999) in their previous reports.

In the present work we have also compared pharmacokinetic properties of carvedilol in normotensive and L-NAME hypertensive rats. Our report demonstrated that administration of L-NAME during 2 weeks does not modify pharmacokinetic behaviour of carvedilol in both awake and anaesthetized animals.

Main objective of the present work was to establish the impact of anaesthesia and L-NAME induced hypertension in the PK–PD parameters of carvedilol cardiovascular response. PK–PD modeling is a powerful tool during preclinical evaluation of drugs, considering that this methodology does not only allow the identification of animal models of efficacy and biomarkers (Höcht et al., 2008; Höcht, Opezzo et al., 2006).

PK–PD parameters of carvedilol chronotropic and antihypertensive effect were scarcely investigated in laboratory animals and human beings. To the best our knowledge, studies regarding PK–PD modeling of pharmacological response to carvedilol in animal models of hypertension are lacking. Conversely, PK–PD properties of the chronotropic and hypotensive effect of carvedilol were evaluated in patients with heart failure and normotensive volunteers, respectively. Tenero Tenero, Henderson, Campanile, Baidoo, and Boyle (2006) have investigated the chronotropic response to S(−)-carvedilol using a direct effect inhibitory E_{max} model, showing that the PK–PD model successfully predicts carvedilol chronotropic response in patients with mild-to-severe heart failure. More recently, hypotensive response to carvedilol was evaluated by means of an effect compartment model in normotensive volunteers (Baek et al., 2008). The authors properly explained time delay in blood pressure reduction by modeling response and carvedilol plasma concentration to a biophase PK–PD model.

In the present work a good relationship between carvedilol plasma concentrations and their chronotropic effect was found in all experimental groups by applying an effect compartment model. Urethane–chloralose anaesthesia selectively increased bradychardic response to carvedilol in L-NAME treated rats, suggesting that anaesthetic mixture increased sensitivity to β-adrenergic cardiac blockade in hypertensive rats. Enhanced bradychardic response to carvedilol is reflected in PK–PD analysis of carvedilol by a greater E_{max} value in anaesthetized L-NAME rats with regard to conscious hypertensive animals. Nevertheless, anaesthesia did not affect estimation of other PK–PD parameter, including EC_{50}, γ and $t_{1/2eq}$ of carvedilol chronotropic response in both control and hypertensive rats. Therefore, it could be concluded that urethane–chloralose anaesthesia only affects PK–PD properties of chronotropic response of carvedilol in L-NAME treated animals without changing parameter estimation in normotensive animals. Increased chronotropic response to carvedilol in L-NAME rats could be explained by the fact that urethane–chloralose anaesthesia increases sympathetic nervous system activity (Carruba et al., 1987; Himori & Ishimori, 1988). Nevertheless, our results also suggest that urethane–chloralose mixture only enhanced cardiac sympathetic activity in hypertensive animals, considering that this anaesthesia did not alter chronotropic response to carvedilol in normotensive animals. Results obtained in normotensive animals are

in accordance with the study of Authier et al. (2008). The authors have evaluated chronotropic response to esmolol, a beta-blocker, in conscious and anaesthetized cynomolgus, finding that anaesthesia does not modify maximal bradychardic response to esmolol.

To the best our knowledge, compromise of β-adrenoceptors in heart rate regulation in L-NAME rats has not been investigated. Our results showed that PK–PD properties of carvedilol are not modified in awake and anaesthetized L-NAME rats with regards to normotensive animals. Therefore, it could be concluded that L-NAME treatment does not modify involvement of cardiac sympathetic activity in heart rate control compared with normotensive animals. It is important to stress out that comparison of PK–PD chronotropic properties of carvedilol between L-NAME hypertensive rats, an animal model of efficacy, with normotensive control group is not affected by the anaesthetic condition.

Urethane–chloralose anaesthesia has been shown to increase sympathetic drive (Carruba et al., 1987; Himori & Ishimori, 1988) and therefore could affect the antihypertensive response to antihypertensive drugs acting on the sympathetic nervous system. To the best our knowledge, impact of urethane–chloralose on PK–PD properties of blood pressure lowering response to sympatholytics drugs have not been evaluated in normotensive and hypertensive animals. In the present work, PK–PD modeling of antihypertensive response to carvedilol has shown that anaesthesia increases efficacy of carvedilol, expressed as E_{max}, but does not affect other PK–PD parameters of the drug in both normotensive and hypertensive L-NAME treated rats. Our results are supported by the fact that urethane–chloralose mixture increased noradrenaline release in sympathetic nervous system efferents (Carruba et al., 1987; Himori & Ishimori, 1988). Although no reports were found regarding the effect of urethane–chloralose mixture on antihypertensive efficacy of beta-blockers, Authier et al. (2008) have found that inhalation anaesthesia increases hypotensive response to esmolol in cynomolgus. Conversely, potency of blood pressure lowering response to carvedilol, expressed as EC_{50}, was not affected by the anaesthetic state in normotensive and hypertensive animals, suggesting that urethane–chloralose does not modify affinity of carvedilol to adrenoceptors.

Previous work by other authors suggested participation of the sympathetic nervous system in the maintenance of the hypertensive state induced by L-NAME administration (Augustyniak, Victor, Morgan, & Zhang, 2006; Biancardi et al., 2007; Pechánová, Dobesová, Cejka, Kunes, & Zicha, 2004). Recently, Biancardi et al. (2007) have found, using ganglionar blockade, that sympathetic tone plays an important role in the initiation and maintenance of hypertension. Our study confirms the involvement of sympathetic nervous system in L-NAME induced hypertension, considering that maximal hypotensive response to carvedilol was significantly greater in conscious and anaesthetized L-NAME rats with regards to control animals. On the other hand, our results also suggested that hypertensive state induced by L-NAME treatment did not modify affinity of carvedilol to α- and β-adrenoceptors, considering that EC_{50} did not differ between control and L-NAME rats.

Finally, it is important to stress out that although urethane–chloralose anaesthesia increased the hypotensive response to carvedilol in both experimental groups, it did not modify conclusions obtained from the animal model of efficacy. In other words, both in conscious and anaesthetized rats, estimated E_{max} for carvedilol hypotensive effect was approximately 2-fold greater in L-NAME animals compared to normotensive rats.

In conclusion, urethane–chloralose anaesthesia is an acceptable experimental condition for the evaluation of PK–PD models of carvedilol, considering that it does not affect the potency of carvedilol for its chronotropic and hypotensive effect. Conclusions obtained from urethane–chloralose anaesthetized animals, regarding the impact of L-NAME treatment on PK–PD properties of carvedilol, did not differ from those obtained from conscious animals. In addition, anaesthesia did not modify pharmacokinetic behaviour of carvedilol in both normotensive and L-NAME hypertensive rats. Although urethane–chloralose increased hypotensive response to carvedilol, PK–PD modeling experiments under this anaesthetic conditions could be useful to establish animal models of antihypertensive efficacy.

Acknowledgements

This work was supported by grant UBACyT B411 from Secretaría de Ciencia y Técnica, Universidad de Buenos Aires, Argentina. Dr. Carlos A. Taira is member of Carrera del Investigador, CONICET, Argentina.

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