

Pharmacokinetic–pharmacodynamic modelling of the hypothermic and corticosterone effects of the 5-HT_{1A} receptor agonist flesinoxan

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Abstract

The current investigation describes the pharmacokinetic–pharmacodynamic correlation of the hypothermic and the corticosterone effect of flesinoxan in the rat simultaneously. A specific objective was to determine the influence of handling the animal. The pharmacokinetic–pharmacodynamic correlation was determined following intravenous administration of 3 and 10 mg/kg flesinoxan in 5 or 15 min. Serial blood samples were obtained for determination of the time course of the flesinoxan and corticosterone concentrations by high performance liquid chromatography. Body temperature was monitored using a telemetric probe. The pharmacokinetics of flesinoxan were described using a three-compartment model. Both the hypothermic and the corticosterone response were successfully described using a physiological indirect response model. It is shown that customizing the animal prior to the experiment has no influence on the pharmacokinetic–pharmacodynamic parameter estimates. Furthermore, the similarity in potency between the hypothermic and corticosterone effects suggests that both are mediated via tissues with a similar receptor–effector coupling efficiency. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Pharmacokinetic–pharmacodynamic modelling; Hypothermia; Corticosterone; 5-HT_{1A} receptor; Flesinoxan; Handling

1. Introduction

Flesinoxan is a new 5-HT_{1A} receptor agonist with improved oral absorption characteristics compared to classical 5-HT_{1A} receptor agonists. In *in vitro* test systems, the drug behaves as a full agonist with an intrinsic activity similar to R-(+)-8-hydroxy-2-(di-*n*-propylamino)tetralin (R-8-OH-DPAT). Flesinoxan exhibits a high selectivity for the 5-HT_{1A} receptor relative to the 5-HT₇ subtype. Furthermore, unlike buspirone for example, *in vivo* flesinoxan is not converted into an active metabolite, which may interfere with the pharmacological activity of the parent compound (Hadrava et al., 1995; Millan et al., 1993; Seletti et al., 1995).

Recently, we have proposed an integrated pharmacokinetic–pharmacodynamic model for the effect of 5-HT_{1A}

receptor agonists using the effect on body temperature as a pharmacodynamic endpoint (Fig. 1; Zuideveld et al., 2001). Briefly, this model is based on the principle of a set-point control in an indirect physiological response model (Dayneka et al., 1993) and is able to characterise quantitatively the effect of 5-HT_{1A} receptor agonists *in vivo*. In this model, the set-point for maintenance of body temperature is attenuated by 5-HT_{1A} receptor agonist via a sigmoidal transducer function. This model has been applied successfully to explain the hypothermic response observed in rats after administration of the 5-HT_{1A} receptor agonists R- and S-8-OH-DPAT in terms of potency and intrinsic activity (Zuideveld et al., 2001). Furthermore, the model has also recently been applied successfully to quantify the effect of the competitive 5-HT_{1A} receptor antagonist *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinyl-cyclohexanecarboxamide (WAY-100,635) *in vivo* (Zuideveld et al., 2002).

The present investigation focuses on the pharmacokinetic–pharmacodynamic correlation of flesinoxan in the temperature model. The primary objective was to deter-

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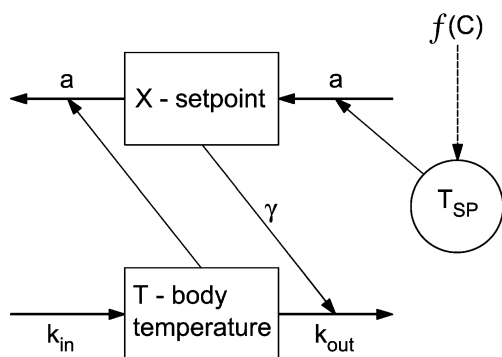


Fig. 1. Full model for describing 5-HT_{1A} receptor mediated hypothermia. The model is based on the concepts of the indirect physiological response model, and takes into account rate constants associated with the warming of the body (k_{in}) and cooling of the body (k_{out}). The indirect physiological response model is combined with the thermostat-like regulation of body temperature, in which body temperature (T) is compared with a fixed reference or set-point temperature (T_{SP}) at rate a , generating a set-point signal X . The extent to which the set-point value decreases is a function of drug concentration $f(C)$, which decreases X by the amplification factor γ . (Reproduced with permission from Zuideveld et al. (2001)).

mine the influence of handling on the pharmacokinetic–pharmacodynamic correlation. A secondary objective was to compare the pharmacodynamics for the effect on body temperature with the effect on corticosterone concentration.

In several studies, a wide range of different pharmacological responses for flesinoxan have been examined (Hadrava et al., 1995; Ahlenius et al., 1991; Groenink et al., 1997). However, very few studies have focused on two effects (i.e. the hypothermic and the corticosterone response) simultaneously (De Koning and De Vries, 1995). Inherent to investigating corticosterone levels in rats, is the need for ‘handling’ in order to maintain a stable corticosterone baseline. It has been shown that corticosterone levels in non-handled rats will increase regardless of any drug being administered (Bassett et al., 1973). Handling the rat a number of times results in a number of corticosterone surges prior to the experiment. It is well established that exposure to corticosterone may influence the level of 5-HT_{1A} receptor expression (Chaouloff, 1993, 1995; Le Corre et al., 1997; Sibug et al., 2000), and thereby the 5-HT_{1A} mediated pharmacological response. Therefore, the present study was specifically aimed at the characterisation of possible pharmacodynamic adaptations due to handling. Furthermore, as the 5-HT_{1A}-mediated increase in corticosterone as well as the hypothermic response can be quantified in terms of intrinsic activity and potency, both effects can be compared.

2. Materials and methods

Experiments were performed on male Wistar rats (Broekman, Someren, The Netherlands) weighing 283 ± 4 g (mean \pm S.E.M., $n=42$) and were approved by the Leiden

University Ethics Committee. The animals were housed in standard plastic cages (six per cage before surgery and individually after surgery). They were kept in a room with a normal 12 h light/dark cycle (lights on at 7:00 a.m. and lights off at 7:00 p.m.) and a temperature of 21 °C. During the light period, a radio was on for background noise. Acidified water and food (laboratory chow, Hope Farms, Woerden, the Netherlands) was provided ad libitum before the experiment.

2.1. Surgical procedure

Eight days prior to the experiment, the rats were operated upon. The animals were anaesthetised with an intramuscular injection of 0.1 ml/kg Domitor (1 mg/ml medetomidine hydrochloride, Pfizer, Capelle a/d IJssel, The Netherlands) and 1 ml/kg Ketalar (50 mg/ml Ketamine base, Pfizer, Hoofddorp, The Netherlands). Indwelling pyrogen-free cannulae for drug administration were implanted into the right jugular vein (polythene, 14 cm, 0.52 mm i.d., 0.96 mm o.d.) for regular infusions of flesinoxan. For blood sampling, the left femoral artery (polythene, 4 cm, 0.28 mm i.d., 0.61 mm o.d.+20 cm, 0.58 mm i.d., 0.96 mm o.d.) was cannulated. Cannulae were tunnelled subcutaneously to the back of the neck and exteriorised. To prevent coagulation of blood, the cannulae were filled with a 25% (w/v) solution of polyvinylpyrrolidone (Brocacef, Maarssen, The Netherlands) in a 0.9% (w/v) sodium chloride solution (NPBI, Emmer-Compascuum, The Netherlands, pyrogen-free) that contained 50 I.U./ml heparin (Leiden University Medical Center, Leiden, The Netherlands). Just before the experiment, the polyvinylpyrrolidone solution was removed and the cannulae were flushed with saline containing 20 I.U./ml heparin. The skin in the neck was stitched with normal suture and the skin in the groin was closed with wound clips. Furthermore, a telemetric transmitter (Physiotel implant TA10TA-F40 system, Data Sciences International (DSI), St. Paul, MN, USA) (weighing ± 7 g), which had been made pyrogen-free with CIDEX (22 g/l glutaraldehyde, Johnson & Johnson Medical, Gargrave, Skipton, UK) for at least 2 h, was implanted into the abdominal cavity for the measurement of core body temperature. After surgery, an injection of the antibiotic ampicilline (ampicilline trihydrate 200 mg/ml, A.U.V., Cuijk, The Netherlands) was administered to aid recovery.

2.2. Experimental protocol

2.2.1. Dosage regimen

Eight days after surgery the experiments were performed. Rats were divided into two groups, 12 rats would undergo a handling procedure and 30 would not. The handling procedure involved accustoming the rats to the investigators, experimental setting and experimental procedures by ‘practicing’ the experiment six times (i.e. days) prior to the actual experiment. Of this group, six rats received 3 mg/kg flesinoxan in 5 min and six received a vehicle treatment.

The other rats received either flesinoxan or the vehicle. Flesinoxan was administered 3 mg/kg in 5 min ($n=6$), 10 mg/kg in 5 or 15 min ($n=6$ per group) infusion. Vehicle infusions consisted of administration of equivalent amounts of saline. For the infusions, an external cannula with a specific volume was filled with a solution of the drug in an amount of saline, which was calculated according to the weight of the rat and connected to the infusion pump (BAS beehive, Bioanalytical Systems USA). All the experiments started at 9:00 a.m.

2.2.2. Blood sampling

At predefined times, two serial blood samples of 50 μ l each were taken to determine both the concentration of the drug and the concentration of corticosterone. Approximately 15 samples were taken according to a fixed time schedule. The exact amount was measured with a capillary and transferred into either a glass centrifuge tube containing 400 μ l of Millipore water for hemolysis (pharmacokinetic samples) or an Eppendorf-vial containing 50 μ l of a 1% EDTA solution in saline (pH 7.4) (corticosterone samples). During the experiment, the samples were kept on ice. After the experiment, pharmacokinetic samples were stored at -20°C pending analysis. The corticosterone samples were centrifuged and 70 μ l of 'plasma' was transferred to a clean tube and stored at -20°C pending analysis.

2.3. Data acquisition

2.3.1. Temperature measurements

To measure the body temperature of the rat, a telemetric system (Physiotel Telemetry system, DSI) was used. A telemetric transmitter (Physiotel implant TA10TA-F40, DSI) was implanted in the abdominal cavity of the rat. The transmitter measured the body temperature every 30 s for a 2-s period and signalled it to a receiver (Physiotel receiver, model RPC-1, DSI). The receiver was connected to the computer through a BCM 100 consolidation matrix (DSI). The computer processed the data and visualised the temperature profiles (Dataquest LabProTM software, DSI, running under OS/2 Warp, IBM) as it did for room temperature (C10T temperature, DSI).

2.3.2. HPLC analysis of flesinoxan

The blood concentrations of flesinoxan were assayed by an enantioselective high performance liquid chromatography (HPLC) method, slightly modified from one described previously (Van der Sandt et al., 2001a). Briefly, detection with the HPLC system was obtained using an electrochemical detector (DECADE, Antec Leyden, Zoeterwoude, The Netherlands) operating in DC mode at 0.63 V, at a temperature of 30°C . Chromatography was performed on a Chiralcel OD-R (Diacel Chemical Industries, Tokyo, Japan). The mobile phase was a mixture of 50 mM phosphate buffer (pH 5.5) acetonitrile (75:25, v/v) and contained a total concentration of 5 mM KCl and 20 mg/l of EDTA (flow,

1 ml/min). The chromatograms were analysed using Millennium-Chromatography Manager (Waters, Milford, MA, USA). The analytes were extracted from blood using two liquid–liquid extraction steps. Briefly, to 50 μ l of blood hemolyzed in 400 μ l of water, 50 μ l of the internal standard DU-28848 was added. After mixing, 2 ml of acetonitrile was added, vortexed and centrifuged. To the supernatant, 0.5 ml 8% acetic acid solution, together with a 2 ml methylisobutylketone/ether (50:50, v/v) mixture was added. After vortexing for 2 min, the organic layer was removed and 1 ml of a 1 M NaOH solution together with 3 ml of dichloromethane was added. After mixing, the water phase was removed and the organic phase was evaporated. The remaining residue was redissolved in 100 μ l of a 20% acetonitrile solution. Calibration curves in the concentration range of 0.1–5000 ng/ml were constructed and analysed with each run by weighted linear regression ($[\text{weight factor}=1/(\text{peak-area ratio})^2]$). Recovery, intra- and inter-assay variations were determined using spiked blood of 50 and 1000 ng/ml. For flesinoxan, the recovery was ($n=3$, mean) 76% over the whole concentration range (external standard method and corrected for loss of volume). Intra-assay variation ($n=4$) was 5.9% and 4.2% (accuracy: 1.8% and 4.5%) for 500 and 1500 ng/ml. Inter-assay variation ($n=10$) was 9.1% and 6.7% (accuracy: 4.0% and -0.03%) for 500 and 1500 ng/ml. Detection limit (signal to noise ratio of 3) using 50 μ l of blood was 0.06 ng/ml.

2.3.3. HPLC analysis of corticosterone

The blood concentrations of corticosterone were assayed by a HPLC method which was slightly modified from a method by Wong et al. (1994). Briefly, detection with the HPLC system was obtained using an UV detector (Spectroflow 757, Kratos Analytical Instruments) operating at 254 nm. Chromatography was performed using a reversed phase C-18 column (microsphere C-18, 3 μ m, 10 cm \times 4.6 mm i.d., Chrompack). The mobile phase consisted of a mixture of acetonitrile/water/acetic acid (35:65:0.05, v/v/v) with a pH of 3. The pump (Spectroflow 400, Kratos analytical Instruments) was set at a flow rate of 1 ml/min and the samples were injected using an autosampler (WISP 712, Waters). Chromatograms were analysed using an integrator (Shimadzu CR3A, Shimadzu Kyoto, Japan). The analytes were extracted from the plasma/saline mixture using a liquid/liquid extraction. To 50 μ l of the plasma mixture, 50 μ l of the internal standard dexamethasone, 5 ml dichloromethane and 0.5 ml 3 M NaOH were added. After vortexing and centrifuging, the water-phase was removed and the organic phase was evaporated at 30°C . The dry residue was redissolved in 100 μ l 25% ethanol, of which 50 μ l was injected into the HPLC system. The plasma used for the calibration curve was produced from blood of untreated rats. All endogenous corticosterone was removed by adding an equal volume of 0.04 g/ml active coal to a volume of plasma. After vortexing for 90 min, the suspension was centrifuged for 60 min at 5000 rpm and the plasma was

decanted and filtered through a 0.2 µm filter (Schleicher and Schuell, Dassel, Germany). Calibration curves in the concentration range of 5–1000 ng/ml were constructed and analysed with each run by weighted linear regression ([weight factor=1/(peak–area ratio)²). Recovery, intra- and inter-assay variations were determined using spiked plasma of 50 and 1000 ng/ml. Recovery was ($n=3$, mean±S.E.M.) 90% over the whole concentration range. Intra-assay variation ($n=4$) was 8.9% and 2.7% (accuracy: 6.7% and –1.9%) for 50 and 1000 ng/ml. Inter-assay variation ($n=10$) was 10.9% and 6.9% (accuracy: 4.6% and –1.8%) for 50 and 1000 ng/ml. Detection limit (signal to noise ratio of 3) using 50 µl of plasma was 5.6 ng/ml.

2.3.4. Chemicals

Solvay Pharmaceuticals (Weesp, The Netherlands) generously donated flesinoxan and its 3-hydroxymethyl analogue, DU-28848 (*N*-[2-[4-[2,3-dihydro-3-(hydroxymethyl)-1,4-benzodioxin-5-yl]-1-piperazinyl]ethyl]-4-fluorobenzamide hydrochloride). Corticosterone and dexamethasone were obtained from Sigma (The Netherlands). Dichloromethane was purchased from Riedel-De Haën (Seelze, Germany). All other chemicals used were of analytical grade (Baker, Deventer, The Netherlands).

2.4. Data analysis

A population approach was utilised to quantify both the pharmacokinetics and pharmacodynamics of flesinoxan. Using this approach, the population is taken as the unit of analysis while taking into account both intra-individual variability in the model parameters as well as inter-individual residual error (Schoemaker et al., 1998). Modelling was performed using the non-linear mixed effects modelling software NONMEM developed by Boeckman et al. (1992) (Version V 1.1, NONMEM project Group, University of California, San Francisco, USA). Individual predictions were obtained in a Bayesian post hoc step.

2.4.1. Pharmacokinetic analysis

The concentration–time profiles of the flesinoxan were best described using a three-compartment pharmacokinetic model,

$$\begin{aligned} V_1 \frac{dC}{dt} &= \text{in} - \text{Cl} \cdot C - \text{Cl}_2 \cdot C + \text{Cl}_2 \cdot C_2 - \text{Cl}_3 \cdot C + \text{Cl}_3 \cdot C_3, \\ V_2 \frac{dC_2}{dt} &= \text{Cl}_2 \cdot C - \text{Cl}_2 \cdot C_2, \\ V_3 \frac{dC_3}{dt} &= \text{Cl}_3 \cdot C - \text{Cl}_3 \cdot C_3, \end{aligned} \quad (1)$$

where C represents the concentration in the central compartment and C_2 and C_3 the concentration in the distribution

compartments. V_1 , V_2 and V_3 are the compartments' volumes of distribution. Cl represents the Clearance and Cl_2 and Cl_3 the inter-compartmental clearance. For an infusion, in is defined by Eq. (2).

$$\begin{aligned} \text{in} &= \frac{\text{Dose}_{\text{inf}}}{T_{\text{inf}}} \quad \text{when } t \leq T_{\text{inf}}, \\ \text{in} &= 0 \quad \text{when } t > T_{\text{inf}}, \end{aligned} \quad (2)$$

where T_{inf} is the duration of the infusion and Dose_{inf} is the total amount of drug infused. The model implemented in NONMEM's ADVAN11 TRANS4 was used. For all models, inter-individual variability on the parameters was modelled by an exponential equation,

$$P_i = \theta \cdot \exp(\eta_i), \quad (3)$$

where θ is the population value for parameter P , P_i is the individual value and η_i is the random deviation of P_i from P . The values of η_i are assumed to be independently normally distributed with mean zero and variance ω^2 . Residual error was characterised by a proportional error model:

$$\text{Cm}_{ij} = \text{Cp}_{ij}(1 + \varepsilon_{ij}), \quad (4)$$

where Cp_{ij} is the j th plasma concentration for the i th individual predicted by the model, Cm_{ij} is the measured concentration, and ε accounts for the residual deviance of the model predicted value from the observed concentration. The values for ε are assumed to be independently normally distributed with mean zero and variance σ^2 . The values for the population θ , ω^2 , σ^2 , are estimated using the first-order method in NONMEM.

2.4.2. Pharmacodynamic analysis

The time course of the hypothermic response and the effect on corticosterone release were analysed on the basis of physiological indirect response models. In these models, the drug–receptor interaction generates a stimulus, which in turn drives physiological processes. This stimulus characterises the drug–receptor interaction in terms of the drugs' intrinsic activity and potency, and can be described by the sigmoidal model $f(C)$,

$$f(C) = S = \frac{S_{\text{max}} \cdot C^n}{\text{SC}_{50}^n + C^n}, \quad (5)$$

where S is the stimulus, S_{max} is the maximum stimulus the drug can produce, C is the drug concentration, SC_{50} is the concentration required to produce 50% of the maximum stimulus and n is the slope factor, which determines the steepness of the curve.

2.4.2.1. Hypothermic response. Recently, we have developed a physiological model to describe the time course of the hypothermic response in vivo following the administra-

tion of 5-HT_{1A} receptor agonists (Fig. 1; Zuideveld et al., 2001). The model features a set-point control that can be attenuated by the drug receptor interaction ($f(C)$) in combination with an indirect physiological response model (Dayneka et al., 1993). This model considers a zeroth order rate constant associated with the warming of the body (k_{in}) and a first-order rate constant associated with the cooling of the body (k_{out}). The thermostat-like regulation is implemented as a continuous process in which body temperature (T) is compared with a fixed reference or set-point temperature (T_{SP}). 5-HT_{1A} receptor agonists elicit hypothermia by decreasing the set-point value in a non-linear manner as a function of their concentration $f(C)$, according to:

$$\begin{cases} \frac{dT}{dt} = k_{in} - k_{out} \cdot T \cdot X^{-\gamma}, \\ \frac{dX}{dt} = a(T_{SP} \cdot [1 - f(C)] - T), \end{cases} \quad (6)$$

in which X denotes the thermostat signal, which is driven by the difference between the body temperature T and the set-point temperature T_{SP} on a time-scale governed by a . Hence, when the set-point value is lowered, the body temperature is perceived as too high and X is lowered. The decreasing signal relates to the drop in body temperature via an effector function $X^{-\gamma}$, in which γ determines the amplification. Raising this function to the loss term $k_{out} \cdot T$ therefore facilitates the loss of heat. With four system parameters to be estimated, the degree of parameterization in Eq. (6) is high and this may lead to parameter identifiability problems. It can be shown that one parameter can be eliminated in a procedure involving redefinition of variables (Zuideveld et al., 2001). The procedure results in the definition of the parameters A and B where,

$$A = \frac{a \cdot T_0}{X_0} = a \left(\frac{k_{in}}{k_{out}} \right)^{\frac{1}{\gamma}} T_0^{1 - (\frac{1}{\gamma})} \text{ and } B = \frac{k_{in}}{T_0}, \quad (7)$$

where T_0 and X_0 are the values for T and X when no drug is present. Hence, four physiological parameters are reduced to three, and parameter unidentifiability is abolished.

As a result of the introduction of dimensionless quantities, the dependent variable T is rescaled to y . The procedure for calculating the redefined y -values on the basis of the observed temperatures is represented in Eq. (8),

$$y = \frac{T - \bar{T}_{min}}{T_{SP} - \bar{T}_{min}}. \quad (8)$$

In Eq. (8), T is the temperature at time t , T_{SP} is the average temperature from the hour prior to drug administration and \bar{T}_{min} is the average minimal temperature of rats receiving a dose of the full agonists R-8-OH-DPAT (33.1 °C, Zuideveld et al., 2001). This temperature is used as the maximal response as defined by S_{max} equals 1 for a full agonist, such as R-8-OH-DPAT.

2.4.2.2. Corticosterone response. The plasma corticosterone concentration–time profiles were described by an indirect physiological response model (Dayneka et al., 1993);

$$\frac{d[\text{Corticosterone}]}{dt} = k_{in} \cdot f(C) - k_{out} \cdot [\text{Corticosterone}], \quad (9)$$

where Corticosterone represents the corticosterone concentration, $f(C)=S$ (Eq. (5)), k_{in} is a zeroth order rate constant associated with the production of corticosterone and k_{out} a first-order rate constant associated with the elimination of corticosterone. The baseline or no-drug level of corticosterone, $[\text{Corticosterone}]_0$, can be defined in terms of k_{in} and k_{out} :

$$[\text{Corticosterone}]_0 = \frac{k_{in}}{k_{out}}. \quad (10)$$

2.4.2.3. Modelling. Both pharmacodynamic models were implemented in NONMEM using ADVAN6. For the modelling of the effect on body temperature parameterisation is different from Eq. (7), where B is purely phenomenological. However, as the individual T_{SP} values are known, the parameter B can be calculated from k_{in} . Therefore, the estimated physiological parameters were k_{in} , A and γ . Initial estimates of the model parameters were obtained by simulation. The observed time dependent variable temperature measurements were redefined as described in Eq. (8). For the effect on corticosterone on basis of Eq. (9), k_{in} was estimated using Eq. (10) since baseline corticosterone concentration was measured. For both models, inter-individual variability on the parameters was modelled to an exponential equation, such as described in Eq. (3). Residual error was characterised by a proportional error model:

$$ym_{ij} = yp_{ij} \cdot (1 + \varepsilon_{ij}), \quad (11)$$

where yp_{ij} is the j th prediction for the i th individual predicted by the model, ym_{ij} is the measurement, and ε accounts for the residual deviance of the model predicted value from the observed value. The values for the population θ , ω^2 , σ^2 , in the set-point model are estimated using the centering first-order conditional estimation method with the first-order model in NONMEM. A conditional estimation method was used due to the high degree of non-linearity of the model and the high density of the data. The centering option gives the average estimate of each element of η together with a P -value which can be used to assess whether this value is sufficiently close to zero. The occurrence of an average η that is significantly different from zero indicates an un-centered or a biased fit. In order to reduce computing time as required with just the conditional estimation method, this method was chosen, and not because the average estimates of each element of η were expected to be different from zero (Boeckman et al., 1992;

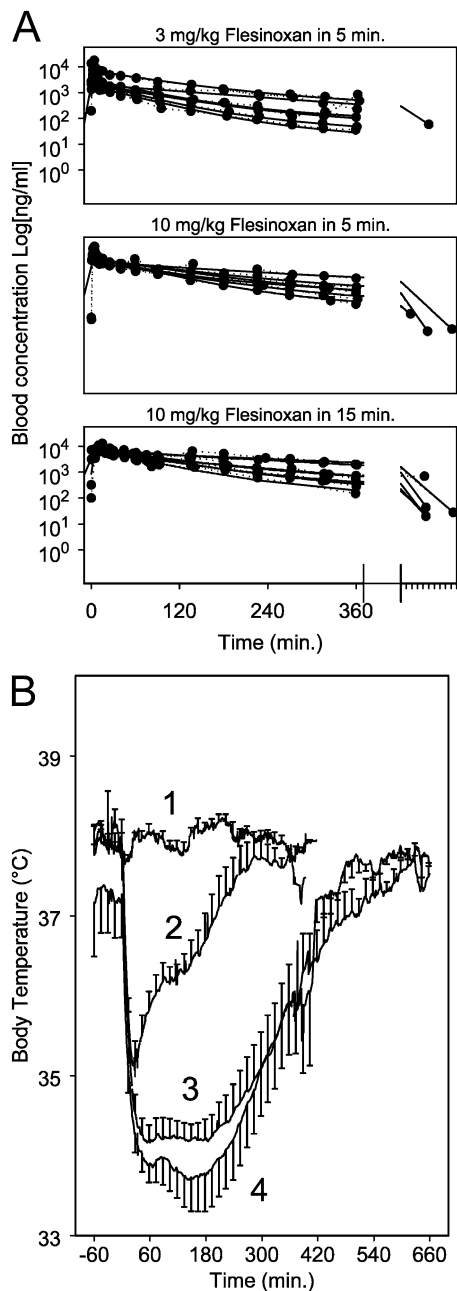


Fig. 2. (A) Pharmacokinetic profiles of fleisinoxan in non-handled rats after administration of 3 mg/kg in 5 min ($n=6$), 10 mg/kg in 5 min ($n=6$) and 10 mg/kg in 15 min ($n=6$). Both the individually measured profiles (dashed lines with markers) and the individual prediction (thick line) are represented. The last tick on the x-axis represents 35 h, the intervals of the ticks after the axis-break is 5 h. (B) Accompanying average temperature–time profiles (\pm S.E.M.) for vehicle treatment (1, $n=12$), the 3 mg/kg in 5 min (2), 10 mg/kg in 5 min (3) and 15 min (4) treatments. All infusions started at $t=0$.

Lindstrom and Bates, 1990). To further decrease computing time only 1/16th of the temperature data was used for modelling, thereby reducing the temperature measurements from over 900 measurements per individual to approximately 60. The implication of this reduction is that there is

a data point every 8 min, as opposed to every 0.5 min. This reduction did not void the integrity of the data profiles. The values for the population θ , ω^2 and σ^2 , for the corticosterone response are estimated using the first-order method in NONMEM.

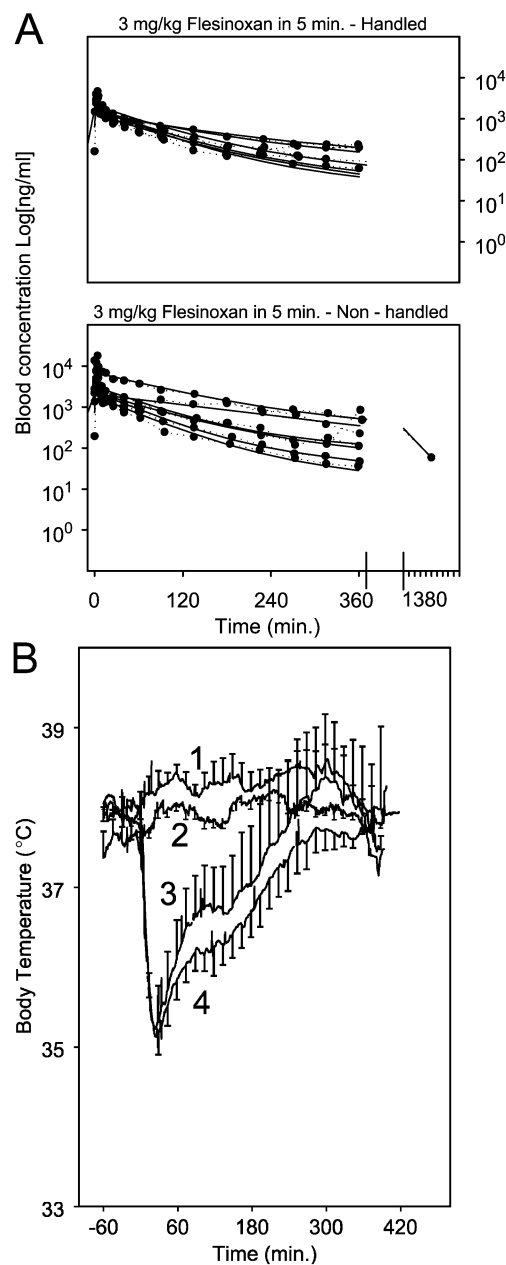


Fig. 3. (A) Pharmacokinetic profiles of fleisinoxan after administration of 3 mg/kg in 5 min in handled and non-handled rats (both $n=6$). Individually measured concentration profiles are represented by dashed lines with markers, whereas the individual predictions are represented by a thick line. The last tick on the x-axis represents 35 h, the intervals of the ticks after the axis-break is 5 h. (B) Accompanying average temperature–time profiles (\pm S.E.M.) for vehicle treatments (1 and 2) and fleisinoxan treatments (3 and 4) for handled (1 and 3) and the non-handled (2 and 4) rats. All infusions started at $t=0$.

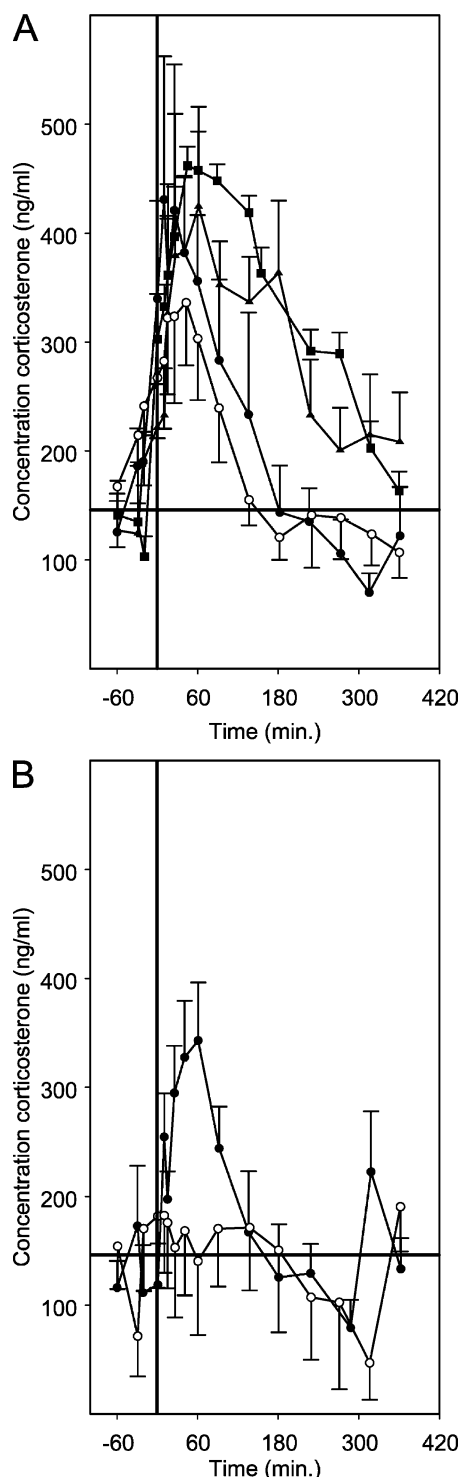


Fig. 4. Average corticosterone–time profiles (\pm S.E.M.) for (A), non-handled rats which had received vehicle treatments (open circles, $n=12$), 3 mg/kg in 5 min (closed circles, $n=6$), 10 mg/kg in 15 min (closed squares, $n=6$) or 15 min (closed triangles, $n=6$) of flesinoxan. (B) Handled rats which had received vehicle treatment (open circles, $n=6$) and 3 mg/kg in 5 min (closed circles, $n=6$). All flesinoxan infusions started at $t=0$.

2.4.3. Statistical analysis

Goodness-of-fit was analysed using the objective function, and various diagnostic methods as present in Xpose version 3.04 (S-plus based model building aid (Jonsson and Karlsson, 1997)). Model selection was based on the Akaike Information Criterion (Akaike, 1974) and assessment of parameter estimates and correlations.

3. Results

Fig. 2 shows the average plasma concentration and hypothermic effect versus time profiles of flesinoxan in non-handled rats following the administration of 3 mg/kg in 5 min ($n=6$), 10 mg/kg in 5 min ($n=6$) and 10 mg/kg in 15 min ($n=6$), respectively. The average baseline temperature (\pm S.E.M., $n=12$) was 37.95 ± 0.004 °C. Following administration of 3 and 10 mg/kg a maximal decrease in body temperature of 3.0 ± 0.44 and 3.8 ± 0.20 °C was observed after approximately 24 and 54 min, respectively. After reaching the temperature minimum, upon administration of 3 mg/kg, a sharp increase followed by a ‘plateau phase’ is observed before the body temperature returns to baseline after approximately 6 h (Fig. 2B). The plateau phase is also observed, but to a lesser extent, after administration of 10 mg/kg, and the effect after reaching the minimum body temperature returns to baseline more gradually after 9 h (Fig. 2B).

Fig. 3 shows the concentration and hypothermic effect versus time profiles following administration of 3 mg/kg in handled and non-handled rats. The average baseline temperature (\pm S.E.M., $n=6$) was 38.24 ± 0.007 °C for the handled rats, which was statistically significantly different from the non-handled ones ($P < 0.0001$). Following administration of 3 mg/kg of flesinoxan ($n=6$) a maximal decrease in body temperature of 3.0 ± 0.27 °C was observed after approximately 28 min. The maximal decrease following a 3 mg/kg administration of flesinoxan between the handled and the

Table 1
Population pharmacokinetic parameters and inter- and intra-individual variabilities of flesinoxan

Drug	Parameter	Value	CV inter-individual (%)	95% C.I.	Unit
Flesinoxan (non-handled and handled)	Cl	2.96	51	2.48–3.43	ml/min
	Cl ₂	0.827	59	0.393–1.261	ml/min
	Cl ₃	48.1	39	39.8–56.4	ml/min
	V ₁	10.2	70	5.06–15.3	ml
	V ₂	226	61	151–301	ml
	V ₃	347	30	305–389	ml

CV intra-individual: 23%

CV inter-individual is the inter-individual coefficient variation, CV intra-individual is the intra-individual coefficient of variation and 95% C.I. is the 95% confidence interval over the precision of the estimated parameter.

Table 2

Population pharmacodynamic parameters and inter- and intra-individual variability's of the hypothermic effect of flesinoxan using the set-point model

Type/Drug	Parameter	Value	CV inter-individual (%)	95% C.I.	Unit
Physiological	k_{in}	2.23	49	1.91–2.55	°C/min
	A	0.0167	76	0.0003–0.0331	min ⁻¹
	γ	6.42	87	–1.02–13.7	n.a.
Flesinoxan	SC ₅₀	714	33	692–736	ng/ml
	n	1.84	63	0.689–0.721	n.a.
	S_{max}	0.705	43	1.81–1.71	n.a.

CV intra-individual: 11%

CV inter is the coefficient of variation and CV intra is the coefficient of the random error. All the handled and non-handled rats were fitted together.

non-handled rats is not statistically significantly different ($P>0.99$) and the time versus effect profiles show a similar plateau phase (Fig. 3B).

The average time–effect profiles for the corticosterone concentrations after administration of vehicle and flesinoxan in both non-handled and handled rats are depicted in Fig. 4. For non-handled rats, the average starting (1 h prior to the infusion) concentration of corticosterone in blood (\pm S.E.M.) was 170 ± 55 ng/ml ($n=12$). Concentration started to rise immediately after the first sample was taken ($t=-60$), reaching a maximal concentration of 340 ± 57 , 430 ± 130 and 460 ± 17 ng/ml approximately 42, 10 and 45 min after the start of the vehicle, 3 and 10 mg/kg infusions, respectively. The increase in corticosterone lasted slightly longer for the 10 mg/kg administration. The average baseline concentration (entire experiment) for the handled rats was 170 ± 12 ng/ml ($n=6$), identical to that observed in non-handled rats. Following administration of 3 mg/kg ($n=6$) in handled rats, a maximal concentration after the start of the infusion of 320 ± 45 ng/ml was observed after approximately 60 min, which is statistically significantly different from baseline ($P<0.01$).

3.1. Pharmacokinetic analysis

During the experiments, blood samples were taken to construct individual concentration–time profiles for flesi-

Table 3

Mean, median, lowest (min) and highest (max) values of the individually estimated post hoc pharmacodynamic parameter estimates for all the non-handled and handled rats

	Non-handled			Handled		
	Median	Mean	min–max	Median	Mean	min–max
k_{in} (°C/min)	2.19	2.46	1.23–6.693	2.27	2.18	1.59–2.83
A (min ⁻¹)	0.017	0.016	0.009–0.022	0.017	0.019	0.016–0.026
γ	6.94	6.78	3.93–9.30	7.05	7.69	6.84–10.7
SC ₅₀ (ng/ml)	526	656	237–1980	555	538	449–608
S_{max}	0.735	0.730	0.350–0.999	0.637	0.604	0.368–0.797
n	1.91	2.55	0.489–7.45	1.93	1.54	0.638–2.30

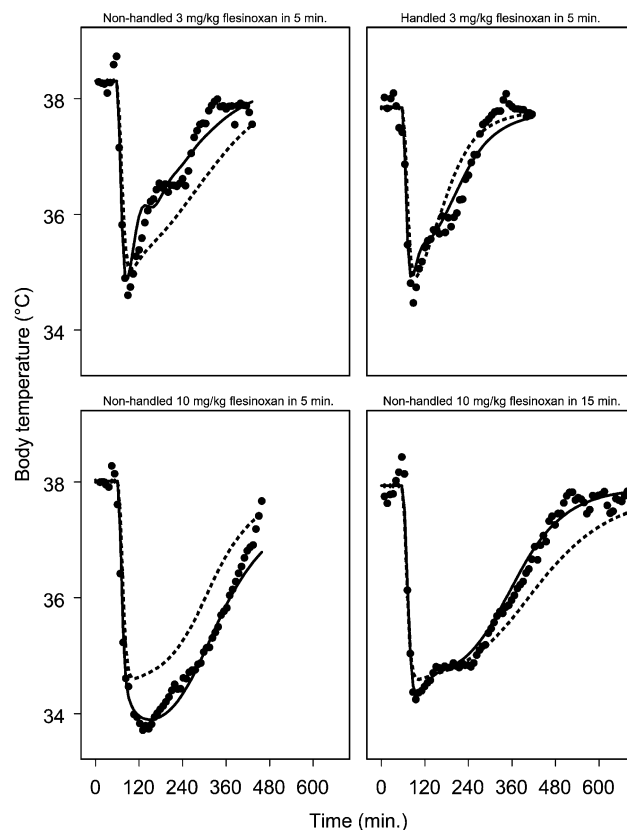


Fig. 5. Selected representative fits of different treatments of flesinoxan both handled and non-handled. Open circles represent measured body temperature, solid line represents individual prediction and the dashed line the population prediction. Infusions all started at $t=60$.

noxan. Individual-based concentration profiles were predicted by fitting the data to a population pharmacokinetic model. On the basis of time–concentration curves, goodness-of-fit-plots and the minimum value of the objective function, a three-compartment model was selected for flesinoxan (Eq. (1)). The individually measured concentration profiles and the individually predicted curves are represented in Figs. 2A and 3A. All parameters were

Table 4

Population pharmacodynamic parameters and inter- and intra-individual variabilities of the corticosterone effect of flesinoxan on handled rats using the indirect physiological response model

Type/Drug	Parameter	Value	CV inter-individual (%)	95% C.I.	Unit
Physiological	k_{out}	0.151	<1	0.0298–0.2752	min ⁻¹
	k_{in}	30.2	n.a.	n.a.	(ng/ml)/min
Flesinoxan	SC ₅₀	462	<1	184–740	ng/ml
	n	2.34	342	1.51–3.17	n.a.
	S_{max}	1.58	126	1.21–1.95	n.a.

CV intra-individual: 42%

CV inter-individual is the coefficient of variation and CV intra-individual is the coefficient of the random error. k_{in} is derived from k_{out} and the baseline value.

estimated in their mixed-effect form, the random effects being incorporated in exponential error models, which are represented in Table 1. The pharmacokinetic parameters were independent of administered dose and the duration of the infusion. Furthermore, there were no differences in pharmacokinetics between the handled and non-handled rats. Table 1 further displays the inter-individual variation, and the intra-individual variation, which were both found to be reasonable. The precision of the parameters predictions, as represented by their 95% confidence intervals, is good.

3.2. Pharmacodynamic analysis

The hypothermic effect–time profiles for both the handled and the non-handled rats receiving flesinoxan were described simultaneously by fitting the set-point model (Eq. (8)) to the data. Potency and relative intrinsic activity of flesinoxan in the hypothermic response were estimated at 714 ng/ml and 0.7, respectively. Parameters were estimated using the centering first-order conditional estimation method with a first-order model. The average values for all η 's were not significantly different from zero. Population parameter estimates are depicted in Table 2. Individual post hoc predictions of the parameters were not found to be biased with respect to the different doses and infusions. Furthermore, no significant differences were found in the individual post hoc estimates between the non-handled and the handled rats as depicted in Table 3. The quality of the fitting of the model to the data is further illustrated in Fig. 5, showing the individual observations, predictions and population predictions for a number of representative rats.

The increase in corticosterone following administration of flesinoxan for the handled rats is described by fitting the indirect physiological response model (Eq. (9)) to the data. Potency and intrinsic activity of flesinoxan in the cortico-

sterone response were estimated at 462 ng/ml and 1.58, respectively. Parameters were estimated using the first-order method. Population parameter estimates are depicted in Table 4. Individual post hoc predictions of the parameters were not found to be biased with respect to the different administrations or treatment groups. The inter-individual variation on the slope factor (n) and maximal stimulus (S_{\max}) is relatively large. Fig. 6 depicts the individual observations, predictions and population predictions for a number of typical rats.

4. Discussion

The objectives of the present investigation were: (a) to determine the pharmacokinetic–pharmacodynamic correlation of flesinoxan using the effect on body temperature as a pharmacodynamic endpoint, (b) to study the influence of ‘handling’ on the pharmacokinetic–pharmacodynamic correlation of flesinoxan, and (c) to compare the pharmacokinetic–pharmacodynamic relationship for the effect on body temperature to the effect on blood corticosterone concentrations.

Both 5-HT_{1A} receptor-mediated hypothermia and an increase in corticosterone are well established and robust markers for 5-HT_{1A} receptor mediated activity (Millan et al., 1993; Cryan et al., 1999; Matheson et al., 1996; Kelder and Ross, 1992). However, despite the fact that numerous studies report these responses for flesinoxan (Hadrava et al., 1995; Seletti et al., 1995; Groenink et al., 1995, 1997) few have studied both responses simultaneously (De Koning and De Vries, 1995). Furthermore, there are no reports of investigations where an integrated pharmacokinetic–pharmacodynamic approach has been applied to obtain parameter estimates characterising the in vivo potency and intrinsic activity on the basis of concentrations rather than doses. One of the complicating factors when studying corticosterone levels is the need for handling of the animals, since corticosterone levels in non-handled rats will increase regardless of the drug being administered (Bassett et al., 1973, and see Fig. 4). It is well known that by handling or accustoming the rats to the investigators and experimental setting, i.e. by rehearsing the experiment a number of times on the days prior to the experiment, corticosterone levels will remain stable during the actual experiment (Bassett et al., 1973 and see Fig. 4). It is our interpretation that handling actually involves stressing the rat a number of times, until the procedure becomes a predictable experience for the rat and hence less stressful. However, stressing the rat a number of times results in a number of corticosterone surges prior to the experiment. As it is well established that changes in corticosterone exposure may influence the level of 5-HT_{1A} receptor expression and thereby potentially the intensity of 5-HT_{1A} receptor mediated responses (Chaoul-off, 1993, 1995; Wissink et al., 2000; Le Corre et al., 1997), the present study also focused on the question of whether

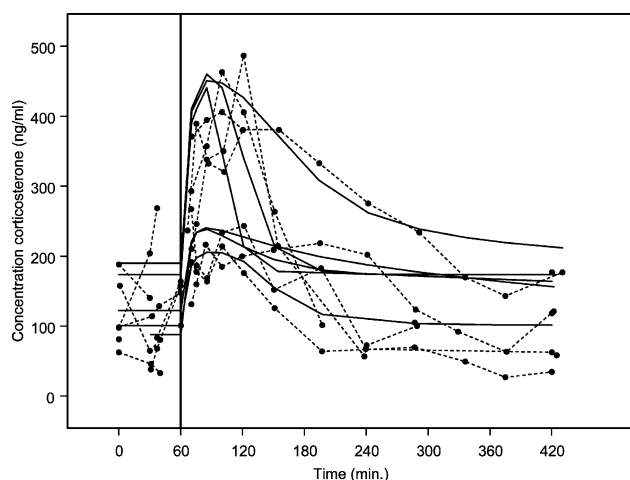


Fig. 6. Selected representative fits of the effect of flesinoxan on corticosterone for handled rats. Both the individually measured profiles (dashed lines with markers) and the population prediction (thick line) are represented. Infusions all started at $t = 60$.

there is a pharmacodynamic difference in temperature response between the handled and non-handled rats.

Upon administration of flesinoxan, a terminal elimination half-life of 136 min is observed. Relative to other 5-HT_{1A} ligands such as R-8-OH-DPAT (86 min (Zuideveld et al., 2001)), buspirone (25 min (Zuideveld et al., unpublished observations)) and WAY-100,635 (33 min (Zuideveld et al., 2002)), this is quite long. This explains the relatively long duration of the hypothermic response after administration of the highest dose. Interestingly, no differences in pharmacokinetic parameter estimates were found between the different doses and infusions of the flesinoxan nor between the handled and the non-handled rats. The observed clearance however is approximately twice as low as values reported previously from our laboratory. In that particular study, rats were only allowed to recover from surgery for 2 days, whereas 8 days of recovery were allowed for rats in this study, allowing full physical recovery (as judged by the restoration of a circadian rhythm in body temperature and locomotor activity). The large difference in pharmacokinetics between these two studies suggests that surgical procedures and experimental setting can have a major influence on the distribution and elimination of flesinoxan.

In order to quantify the pharmacodynamic properties of flesinoxan, the hypothermic response and in the case of the handled rats also the corticosterone response were chosen as pharmacodynamic endpoints. The hypothermic response behaves as an ideal bio-marker, as it is continuous, reproducible, sensitive enough to discriminate between a full and a partial agonist (Hadrava et al., 1995) and selective (Millan et al., 1993; Cryan et al., 1999). We have recently developed an integrated pharmacokinetic–pharmacodynamic model which characterises 5-HT_{1A} receptor-mediated hypothermia in terms of potency and intrinsic activity (Zuideveld et al., 2001). This model features a set-point control in combination with an indirect response model. As it is believed that the 5-HT_{1A} receptors play a role in maintaining the body's set-point temperature (Lin et al., 1983; Schwartz et al., 1998; Zeisberger, 1998), and numerous reports suggest this set-point is regulated through an interplay between the 5-HT_{1A} (hypothermia) and the 5-HT_{2A/C} (hyperthermia) receptor system (Gudelsky et al., 1986; Salmi and Ahlenius, 1998) this model is considered to reflect its physiology. The set-point model allows 5-HT_{1A} receptor agonists to attenuate the set-point via a sigmoidal transducer function, allowing for the estimation of potency and intrinsic activity. An important feature of the model is that it allows characterisation of the complex hypothermic time profiles that are typically observed upon the administration of 5-HT_{1A} receptor agonists. For a full agonist, the model predicts that the typical 'plateau phase' that is observed with a low dose disappears with increasing dosages, as the oscillations associated with the regulatory mechanism become 'over-damped' (Zuideveld et al., 2001). This typical behaviour is also observed for flesinoxan, which therefore behaves as a full rather than a partial agonist. However, since slight

damping is observed and the absolute decrease in body temperature is not as large as for example R-8-OH-DPAT's (4 ± 0.3 °C; Zuideveld et al., 2001), it is estimated as a partial agonist despite the fact that its effect is near maximal. The parameters denoting the physiological part of the model are highly similar to those estimated for R- and S-8-OH-DPAT and buspirone in previous studies (Zuideveld et al., 2001, 2002). Interestingly, the pharmacodynamic parameters show that flesinoxan has a lower potency compared to, for example, R-8-OH-DPAT (potency of 40 ng/ml (Zuideveld et al., 2001)) or buspirone (20 ng/ml (Zuideveld et al., unpublished observations)). In vitro affinities at the 5-HT_{1A} receptor are similar with K_D values of flesinoxan of 8.8 nM compared to 8.4 nM for R-8-OH-DPAT and 7.8 nM for buspirone (Van Steen, 1996). Hence, there does not appear to be a direct correlation between potencies found in vivo and affinities found in vitro. This may in part be explained by active transport processes at the blood–brain barrier. Previous studies from our laboratory have shown that P-glycoprotein efflux pumps may affect the equilibration between blood and the brain (Van der Sandt et al., 2001), and it is conceivable that estimating the potency based on blood concentration results in an underestimation as compared to the other 5-HT_{1A} receptor agonists. However, other possibilities, such as a different receptor reserve or different receptor functionality for 8-OH-DPAT and buspirone in vivo for or on the 5-HT_{1A} receptor should not be excluded.

Despite the fact that a significant difference in baseline temperature was observed between the handled and the non-handled group, no differences were observed in either the physiological or pharmacodynamic parameters (see Table 3 for the mean, median, lower and upper values). This indicated that after 1 week of handling, with the associated surges in corticosterone, the net 5-HT_{1A} receptor function involved in the hypothermic effect had not changed. The change in baseline temperature was not expected, although higher baseline temperatures for rats which had been handled have been reported previously (Michel and Cabanac, 1999). Whether the change in baseline temperature is associated with a changed 5-HT_{1A} receptor function is not known. One could speculate however that 5-HT release in the synaptic cleft has changed due to an altered receptor function of for example the pre-synaptic 5-HT_{1A} receptor.

The observed absolute corticosterone concentrations for baseline levels of handled and non-handled rats are significantly larger than values reported by several other studies (Sibug et al., 1998, 2000; Fagin et al., 1983). It appears that methodological factors play a role here. In the majority of studies reported in the literature, corticosterone is measured with either a commercially available or a 'homemade' radioimmunoassay (RIA). In the present investigation, however, a specific HPLC method has been used for the quantification of the corticosterone concentrations. Corticosterone concentrations reported by other authors using HPLC assays are very similar to ours (Haughey and Jusko, 1988; Wong et al., 1994). Furthermore, 'validation' of our

results with those obtained using a commercially available RIA kit did not yield a line of unity (results not shown). Since the HPLC assay had been validated using a mass spectrometer (Wong et al., 1994) and was sensitive enough for this study, it was our method of choice.

In the modelling of the corticosterone effects, only data from handled rats was considered, since in ‘non-handled’ rats, a profound baseline drift in corticosterone concentrations is observed. Theoretically, it is possible to incorporate such a change in the pharmacokinetic–pharmacodynamic model, but this requires a mechanistic understanding of the placebo response and the drug effect. In this respect, both additive and more complex interaction models need to be considered. At present, no specific information is available regarding this interaction. At present, this makes it impossible to come up with a realistic model for the simultaneous analysis of the data from handled and ‘non-handled’ rats. Interestingly, the rise in corticosterone level as observed after administration of the 10 mg/kg dose of flesinoxan does last significantly longer, than following 3 mg/kg, which is consistent with the behaviour of an indirect physiological response model (Dayneka et al., 1993). The increase in corticosterone levels in the handled rats as a result of the 3 mg/kg administration of flesinoxan was described using the indirect physiological response model. The value of the rate constant associated with the elimination of corticosterone from blood (k_{out}) is considerably shorter than the elimination half-life of corticosterone as obtained on the basis of analysis of the serum concentration versus time profile as reported by other authors; 4.6 min ($\ln 2/k_{out}$) versus 25 min (Sainio et al., 1988). In theory, these values should be identical, and a comparison of these values could therefore serve as a validation of the indirect physiological response model (Van Schaick et al., 1997). With regards to the reported value of the elimination half-life of 25 min, it is important that this value was estimated after the administration of a large dose of exogenous corticosterone, without considering the endogenous corticosterone concentration (Sainio et al., 1988). This may explain why the terminal elimination half-life of corticosterone has been overestimated in this previous investigation.

In the present investigation, the effects of flesinoxan on both the lowering of body temperature and the corticosterone response have been quantified on the basis of blood concentrations. This makes it possible to compare the pharmacodynamic parameter estimates for the two effects. Interestingly the potency estimate for the rise in corticosterone concentration is very similar to the potency found in the hypothermic response. This suggests that the same tissue or tissues with very similar a receptor densities and efficiency of the receptor–effector coupling might mediate both responses.

In the present study, the effect of flesinoxan on body temperature and on corticosterone was characterised in an integrated pharmacokinetic–pharmacodynamic analysis. The hypothermic response was described using the recently

developed physiological set-point model. Despite the fact that the baseline temperature of handled rats was significantly increased, no differences were found in the hypothermic response mediated by flesinoxan, nor in the pharmacodynamic parameter estimates. This indicates that ‘handling’ has only minor effects on 5-HT_{1A} receptor function in vivo. The time course of increase in corticosterone as observed after administration of flesinoxan was successfully described using the indirect physiological response model for the handled rats. The potencies found between the hypothermic and the corticosterone effect suggests that both effects are mediated by similar—if not the same tissues.

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