The 'free drug hypothesis' : fact or fiction?

Steeg, T.J. van

Chapter 6

Reproducible and time-dependent modification of serum protein binding in Wistar Kyoto rats

TJ van Steeg[1], EHJ Krekels[1], M Danhof[1] and ECM de Lange[1,2]

[1]Division of Pharmacology, LACDR, Leiden University, Leiden, Leiden, The Netherlands
[2]Brains Online, Groningen, The Netherlands

J. Pharmacol. Toxicol. Methods (2007) 56: 72-78

Summary

Purpose and Background The theoretical basis of the influence of (alterations in) plasma protein binding on pharmacokinetics (PK) is well-established. In contrast, the impact of protein binding on pharmacodynamics has not been examined in a systematic manner. Here we present an experimental approach to modify serum protein levels and binding in the rat, in a robust, reproducible, and time-dependent manner.

Methods Male Wistar Kyoto rats were divided into three different groups. The control group (n=4) did not receive treatment. In the cannulation(-) group (n=6) the rats were instrumented with three permanent blood cannulas. The rats in the cannulation(+) group received in addition to the cannulation a subcutaneous injection of turpentine oil of 100 μl/100 g bodyweight. The effects were characterised in terms of 1) the time course of serum levels of albumin and α_1-acid glycoprotein (AGP), and 2) the effect on the *ex-vivo* serum protein binding of S(-)-propranolol.

Results In control rats the AGP serum concentration was stable at a value of 169 ± 16 μg/ml. In the cannulation(-) group a maximum ten- to fifteen-fold increase in serum AGP concentration was observed at 48 hours post-surgery, followed by a gradual return back to baseline within one week. In the cannulation(+) group a similar concentration-time profile for AGP was found, but without a complete return to baseline within one week and with a much higher variabilty. *Ex-vivo*, an increase in AGP serum concentration from 55 to 675 μg/ml resulted in a profound decrease in the free fraction of S(-)-propranolol from 14 ± 0.6 to 1.9 ± 0.3 %.

Conclusions In conclusion, through cannulation alone the serum protein levels and binding were modified in a robust, reproducible and time-dependent manner. Therefore this experimental approach is suitable for the investigation of the influence of protein binding on both pharmacokinetics and pharmacodynamics.

6.1 Introduction

The binding of drugs to plasma proteins can have a major influence on the pharmacokinetics (PK) and pharmacodynamics (PD). At present the theoretical basis of the influence of (alterations in) serum protein binding on the PK is well established and clearly defines protein binding as restrictive or non-restrictive, depending on well-specified conditions (Rowland and Tozer, 1995). Although some studies indicate both restrictive and non-restrictive properties for the PD, it is generally assumed that only the free concentration in plasma is responsible for the pharmacological effect of drugs ('free drug hypothesis') *in vivo*.

For certain drugs like benzodiazepines, opiates, and steroids experimental data indicate that it is indeed the free concentration that determines the intensity of the response (Cox *et al.,* 1998; Derendorf *et al.,* 1993; Mandema *et al.,* 1991). Interestingly, for other drugs such as A_1 adenosine agonists, it appears that it is the total rather than the free concentration that determines the response (Van Der Graaf *et al.,* 1999). Moreover, for certain drugs such as propranolol, the fraction that is transported into tissues is reported larger than the free fraction in serum indicating that protein binding may be non-restrictive for the pharmacological effect of these drugs (Pardridge and Landaw, 1984). These results imply the need for a thorough investigation of the 'free drug hypothesis' *in vivo*.

In order to test the 'free drug hypothesis' in a systematic manner, PKPD investigations on the influence of protein binding on *in vivo* drug effects are needed, under experimental conditions in which the levels of proteins and thereby the extent of protein binding can be varied. To that end, the objective of this investigation was to develop an *in vivo* experimental approach to alter serum protein binding in rat, in a

robust and reproducible manner,

In blood, drugs bind to serum albumin, α_1-acid glycoprotein (AGP) (Kremer *et al.,* 1988), and other blood constituent like lipoproteins, erythrocytes and α-, β-, γ-globulins (Wright *et al.,* 1996). Albumin is the most important drug-binding protein due to its high concentration in blood. Albumin has several high- and low-affinity binding sites and is mainly involved in the binding of acidic drugs (Day and Myszka, 2002; Muraikushiya *et al.,* 1993; Notarianni, 1990; Piafsky, 1980). AGP is mainly involved in the binding of neutral and basic drugs (Kopecky *et al.,* 2003; Kremer *et al.,* 1988). Especially AGP is of interest as its levels are susceptible to changes.

Under physiological conditions the serum AGP concentration in the rats is approximately 200 $\mu g\ ml^{-1}$, while in human it ranges from 400 - 1000 $\mu g\ ml^{-1}$ (Kopecky *et al.,* 2003; Kremer *et al.,* 1988). It is known that infection, inflammation or severe injury induces a cascade of reactions called the acute phase response, in which plasma concentrations of AGP increase about ten-fold (Fournier *et al.,* 2000; Muraikushiya *et al.,* 1993; Kushner, 1982).

Over the years, several investigations have reported alterations in plasma protein concentrations in experimental animals (Fournier *et al.,* 2000; Yasuhara *et al.,* 1985; Terao and Shen 1983). Yasuhara et al. reported a 6-fold increase in AGP levels following a laparotomy in rats two days post surgery found time-dependent changes in albumin levels and in the α_1-globulin fraction upon implantation of an indwelling venous catheter. However in this study the number of samples taken was limited while no information was obtained about the specific AGP levels, since merely the overall concentration α_1-globulin fraction was determined.

A rise in AGP content can also be obtained by inducing an acute phase reaction with a subcutaneous or intramuscular injection of turpentine oil (Muraikushiya *et al.,* 1993; Chauvelot-Moachon *et al.,* 1988; Belpaire *et al.,* 2002; Jauregizar *et al.,* 2001; Drechou *et al.,* 1989). However none of these studies reported in a strictly quantitative and reproducible manner the time-dependent changes in plasma protein concentrations.

We studied the time course of the change in serum albumin and AGP concentrations following surgical implantation of permanent arterial and venous cannulas alone, or in combination with a subcutaneous injection of turpentine oil. The effect of elevated levels of AGP on the extent of serum protein binding was determined *ex vivo*. The highly protein bound β-blocker S(-)-propranolol was used as a model drug.

6.2 Methods

Animals

The Animal Ethical Committee of Leiden University approved the use of the animals for the current study protocol under UDEC no. 04027. Male Wistar Kyoto rats (290 g ± 17, n=16) obtained from Janvier (Le Genest Saint Isle, France) were used. Prior to the surgery the rats were acclimatised for at least 5 days at 21°C. The rats had *ad libitum* access to acidified water and food (Laboratory Chow, Hope Farms, Woerden, The Netherlands).

Compounds and Drugs

S(-)-propranolol, pindolol and turpentine oil were purchased from Sigma-Aldrich BV (Zwijndrecht, the Netherlands). Ketanest-S® ((S)-ketaminebase) was purchased from Parke-Davis (Hoofddorp, The Netherlands). Dormitor® (medetomidine hydrochloride) was obtained from Pfizer (Capelle a/d IJssel, The Netherlands). Polyvinylpyrrolidone (PVP) was obtained from Brocacef (Maarsen, The Netherlands).

Heparin (20 IU/ml) was obtained from the LUMC (Leiden University Medical Center) Pharmacy (Leiden, The Netherlands) and 0.9% saline from B. Braun Melsungen AG. (Melsungen, Germany).

Alteration of serum protein levels in rats

Surgery

The rats were anesthetised with a subcutaneous injection of 0.1 ml/100 g Ketanest-S® and an intramuscular injection of 0.01 ml/100 g Dormitor®. During surgery the rats were placed on a heating pad to maintain body temperature at 37 °C. The rats in the treatment groups were instrumented with three indwelling blood cannulas (Portex Limited, Hythe, Kent, England), one in the right jugular vein (Polythene 14 cm, ID 0.58 mm, OD 0.96 mm) and one in both the left and the right femoral artery (Polythene, 4 cm ID 0.28 mm, OD 0.61 mm + 20 cm ID 0.58 mm, OD 0.96 mm). The blood cannulas were subcutaneously tunnelled and externalised at the dorsal base of the neck. To prevent blood clotting, the arterial cannulas were filled with a 25% (w/v) polyvinylpyrrolidone (PVP) solution in a 0.9 % (g/v) saline solution containing 20 IU ml^{-1} heparin. The venous cannula was filled with a saline solution containing 20 IU ml^{-1} heparin.

Experimental Design

The rats were divided into three groups dependent on treatment. 1) control group (n=4) which received no treatment, 2) the cannulation(-) group (n=6), which received surgical implantation of cannulas as described above and 3) the cannulation(+) group (n=6) which recieved in addition to the implantation of cannulas, a single subcutaneous injection of turpentine oil of 100 µl/100 g bodyweight. The turpentine oil was injected immediately after the surgery while the rats were still anesthetised.

To avoid a possible influence of anaesthesia and/or surgery, the control rats did not receive any treatment prior to bloodsampling. Blood samples in control rats were obtained via the tail vein and a total number of six samples of 100 µl were taken during one week. In the cannulation (-) and cannulation (+) group a blood sample was taken during surgery to determine baseline concentrations of alpha-1-acid glycoprotein (AGP) and rat serum albumin (RSA). In the cannulation (-) and cannulation (+) group the blood samples were obtained via one of the arterial cannulas. A total of ten samples (100 µl) were taken during 7 days following treatment. Before taking the first blood sample, the PVP/heparin solution in the cannula was discarded and replaced by a saline solution containing 20 IU ml^{-1} heparin.

Serum was obtained from the blood samples after clotting by 10 min centrifugation (5000 rpm) and frozen at -80 °C until analysis.

Bioanalysis of RSA and AGP in serum

Albumin (RSA)

Quantitative albumin analysis was based on the Albumin Blue 581 dye-binding method as described by Kessler et al. 1997 (Kessler *et al.,* 1997). The calibrator diluent and reagent A and B for the assay reagent were purchased from Fluka (Zwijndrecht, The Netherlands). Rat serum albumin (RSA) was purchased from Sigma-Aldrich BV (Zwijndrecht, The Netherlands). Briefly, a calibration curve (10-200 µg ml^{-1}) was prepared fresh each day by diluting an albumin stock solution (2 mg ml^{-1}) with calibrator diluent. Serum samples were diluted to concentrations within the range of the calibration curve. A volume of 0.5 ml sample or calibration solution was mixed thoroughly with 2.5 ml assay reagent and the relative fluorescence emission intensity was measured immediately (excitation, 600 nm; emission, 630 nm).

Alpha-1-acid glycoprotein (AGP)

AGP concentrations were determined by a single radial immunodiffusion assay using a commercially available kit (Tridelta Development Ltd., Wichlow, Ireland). In short, 5 μl serum or standard solution was added to a well in an agar gel plate containing antibodies against (rat specific) AGP. The plate was placed in a humidified chamber of 37 °C for approximately 24 hours after which the AGP concentration was determined.

Ex-vivo serum protein binding of S(-)-propranolol

Blood was collected from rats by decapitation, pooled, and centrifuged after clotting (10 min, 5000 rpm) to obtain serum for the *ex vivo* protein binding experiments. Serum with normal protein concentrations was collected from control rats (n=3). Serum with increased AGP concentrations was obtained from rats which had been implanted with three permanent cannulas as described previously (surgery section). Blood was collected post surgery at two (n=2) or four (n=1) days.

Experimental Design

The free fraction of S(-)-propranolol was determined by ultrafiltration using the Centrifree® system (Millipore BV, Amsterdam, the Netherlands). Briefly, one day prior to the experiments the filters of the Centrifree® devices were rinsed with 1 ml 0.1M NaOH solution followed by 1 ml deionized water to remove the glycerin preservative in the filter that interfered with the quantitative S(-)-propranolol analysis. Filters were kept wet overnight and dried shortly before the actual experiment. Serum (or RSA-solution) was spiked with the drug solutions and vortexed for 30-60 min at a temperature of 37°C. An aliquot of the mixture was placed in the sample compartment of the ultrafiltration device. The devices were centrifuged at 2000g (2 min, 37°C). Finally the concentration S(-)-propranolol was determined in the filtrate. Control experiments indicated that approximately 30% of the drug binds to the membrane of the ultrafiltration device and therefore the following equation was used to calculate the free fraction.

$$fu = \frac{C_{filtrate}}{0.7 * C_{initial}} \qquad (1)$$

The ultracentrifugation experiments were performed in triplicate or quadruplicate. Serum for the ultrafiltration experiments was obtained from control and cannulated rats as described above. Prior to the ultrafiltration experiments the AGP concentrations in serum were determined. The AGP concentrations in serum, collected from separate rats which received surgical implantation of cannulas alone, were 110 (control), 650 (four days post surgery), 960 and 1350 μg ml^{-1} (two days post surgery). The protein binding of S(-)-propranolol was determined in serum with normal and elevated serum AGP concentrations. An equal volume of propranolol solution (600 ng ml^{-1} in 0.9% saline) was added to the serum and therefore the final propranolol concentration was 300 ng ml^{-1}. The final AGP concentrations in the ultracentrifugation experiments were 55, 325, 480 and 675 μg ml^{-1}. Control experiments were performed in RSA solutions (35 mg ml^{-1}) to determine the contribution of albumin to the overall protein binding of S(-)-propranolol. The concentration S(-)-propranolol in these experiments was 300 ng ml^{-1}.

S(-)-Propranolol Analysis

The HPLC-system consisted of a LC-10AD HPLC pump (Shimadzu, 's Hertogenbosch, The Netherlands), a Waters 717 plus autosampler (Waters, Etten-Leur, The Netherlands), and a FP 920 fluorescence detector (Jasco Co, Tokyo, Japan) with an excitation wavelength of 230 nm and an emission wavelength

of 340 nm. Chromatography was performed on Spherisorb ODS-2 3 µm column (4.6 mm I.D. x 100 mm) (Waters, Millford, MA, USA) equipped with a refill guard column (2 mm I.D. x 20 mm) (Upchurch Scientific, Oak Harbor, WA, USA) packed with pellicular C18 (particle size 20-40 µm) (Alltech, Breda, The Netherlands). The mobile phase consisted of 35% (v/v) 0.1 M sodium acetate buffer (pH 3.4) containing 5 mM octane-sulfonic acid (OSA) and 65% (v/v) acetonitrile. Samples were spiked with an equal volume of internal standard (pindolol) solution and 50 µl was injected into the HPLC-system.

Statistical data analysis

Statistical analysis of the serum AGP and RSA data in the treatment groups compared to the control group was performed using a one-way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparison test for differences between more than two groups. In addition the data were analysed using a two-way analysis of variance to determine the significance of time, treatment and the interaction between both effects.

In the *ex vivo* ultrafiltration studies all samples were compared to the control sample (normal AGP) using the two-tailed unpaired students *t*-test. Statistical tests were performed using InStat version 3.0 for windows (GraphPad, San Diego, USA) and S-PLUS 6.0 (Insightful Corp., Seattle, WA, USA). All data are presented as mean values ± SEM and $p<0.05$ was considered significant.

6.3 Results

Time course of AGP and RSA plasma (of serum) concentrations in rats

AGP serum concentrations

The individual concentration-time profiles for AGP in the 3 treatment groups are shown in figure 1.

The AGP serum concentrations in the control rats remained stable at baseline values of 169 ± 16 µg ml^{-1} throughout the experiment. A t=0 a small but statistically significant difference in AGP concentration was observed between the control group and both the cannulation(-) and cannulation(+) group ($p<0.05$). A large increase in serum AGP concentrations was observed in the rats of the cannulation(-) and cannulation(+) groups. In the cannulation(-) group a maximum ten- to fifteen fold increase in serum AGP concentrations was observed at 48 hrs post surgery. The concentrations gradually returned back to

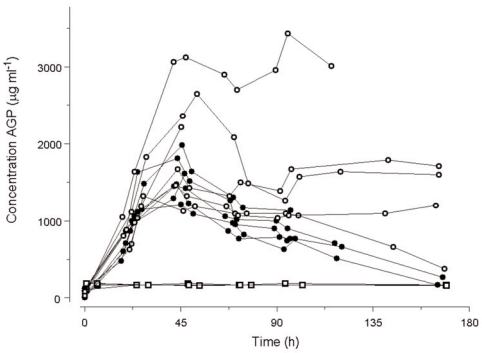

Figure 1, Individual serum concentrations of AGP in control rats (□) in cannulation(-) rats (●) and in cannulation(+) rats (○).

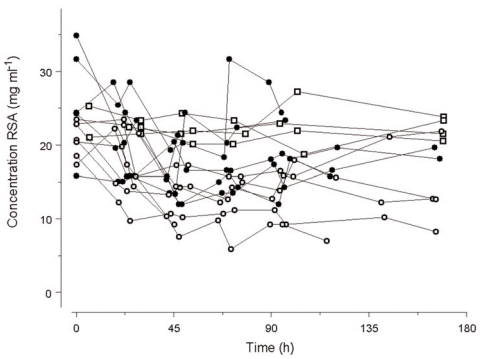

Figure 2, Individual serum concentrations of RSA in control rats (□), in cannulation(-) rats (●) and in cannulation(+) rats (○).

baseline within one week post-surgery. At one, two, three and four days post surgery the difference between the control and the cannulation(-) group was statistically significant (one-way ANOVA, $p<0.05$). On day five and seven the difference was no longer significant. The profiles of the cannulation(+) rats displayed a larger inter-individual variability when compared to the cannulation(-) rats. Maximal AGP concentrations in this treatment were observed 48 hour post surgery. The values of the AGP concentration appeared to be slightly higher compared to the values in the cannulation(-) group, but the difference did not reach statistical significance. The AGP concentrations in the cannulation(+) group remained elevated ($p<0.05$) throughout the experiment. The results of the two- way ANOVA are shown in table I and a significant interaction between the time and treatment effect is observed ($p<0.001$).

Table I, Time and Treatment effect on AGP oncentration (two-way ANOVA)

	Df	SS	MS	F	p-value
Time	5	13618017	2723603	16.298	0*
Treatment	2	17662503	8831252	52.847	0*
Time x Treatment	10	6818213	681821	4.08	0.0002*
Residuals	67	1196297	167109		

** p<0.001*

Table II, Time and Treatment effect on RSA concentration (two-way ANOVA)

	Df	SS	MS	F	p-value
Time	5	13618017	2723603	16.298	0*
Treatment	2	17662503	8831252	52.847	0*
Time x Treatment	10	6818213	681821	4.08	0.0002*
Residuals	67	1196297	167109		

** p<0.001, ** p<0.01*

RSA serum concentrations

In addition to the AGP concentration, the concentration-time course of serum albumin (RSA) was determined. The individual time-concentration profiles for serum RSA following the different treatments are displayed in figure 2. In the control rats the RSA levels remained constant throughout the experiment (comparison with t=0). A decrease in RSA concentrations was observed in the cannulation(-) rats, however the observed decrease was not significantly different from control rats. In the cannulation(+) group, however, the decrease in RSA remained statistically significant up to five days post treatment ($p<0.05$). The comparison of the cannulation(-) and the cannulation(+) groups did not yield any significant differences throughout the experiment ($p>0.05$). The results of the two-way ANOVA are shown in table II. Although time and treatment are significant ($p<0.01$), no significant interaction is observed between these two effects.

Ex-vivo determination of the free fraction of S(-)-propranolol with altered serum AGP levels

The free fraction of S(-)-propranolol was determined in rat serum with AGP concentrations ranging from 55 to 675 μg ml^{-1}. In these serum samples a reduction in the free fraction of S(-)-propranolol from 14 ± 0.6 to 1.9 ± 0.3 % was observed. The relationship between the serum AGP concentration and the serum protein binding of S(-)-propranolol was non-linear. The free fraction of S(-)-propanolol is plotted as function of the reciprocal of the AGP concentration (Figure 3).

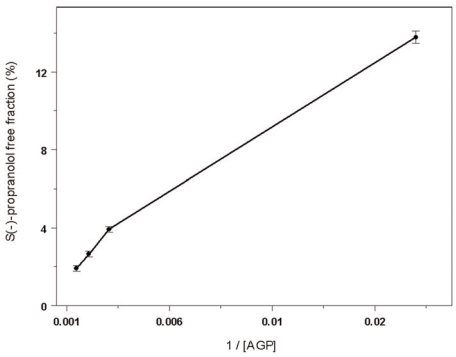

Figure 3, S(-)-propranolol free fraction (%) with increasing AGP concentrations in serum; Each point represents the mean ± SEM (n=4).

6.4 Discussion and conclusion

For the purpose of systematic investigations on the role of free drug concentrations as a determinant of the PKPD relationship of highly protein bound drugs, it is important to modulate the free fraction *in vivo* in a controlled manner. In this study it was shown that cannulation either alone or in combination with a turpentine oil injection result in a robust time dependent change in serum AGP concentration. Subsequently it was found that increased AGP levels resulted in a decrease in the free fraction of the highly bound model drug, S(-)-propranolol. The effect of cannulation combined with a turpentine oil injection on the AGP concentration is somewhat larger than cannulation alone. The variability however, is also larger in the combined treatment and therefore cannulation alone is preferred as experimental approach for the elevation of serum AGP levels *in vivo*.

Previously, Yashuhara (Yasuhara *et al.,* 1985) studied the impact of changes in AGP levels on the PKPD of propranolol. However, in their experimental design, no distinction could be made between the influence of anesthesia, cannulation and the laparotomy on the PKPD relationship of propranolol. Such experimental design would never allow us to investigate of the impact of changes in protein binding on the PKPD relationship at normal AGP levels in conscious rats. Thus, our aim was to establish an experimental rat model for well-characterised increases in serum protein levels with a return to baseline.

In our studies, the basal levels of AGP were found to be 165 ± 19, 85 ± 21 and 55 ± 13 $\mu g\ ml^{-1}$ in the control, the cannulation(-) and the cannulation(+) group respectively. The somewhat higher baseline AGP concentrations as found in the control rats compared to cannulated rats ($p<0.05$) might be caused by the fact that blood samples were obtained from the tail vein in control rats and from the femoral artery in cannulated rats or the result of anesthesia in the cannulation groups. It is known that for some substances, the distribution throughout the body is not completely homogenous and differences exist in arterial and venous concentrations (Frape 1993; Harris *et al.,* 1962; Spanner *et al.,* 1976; Wagner *et al.,* 1990). Our results are similar to what has been found in literature. Under physiological conditions, AGP serum concentrations vary between 100 and 200 $\mu g\ ml^{-1}$ depending on rat strain and experimental conditions (Yasuhara *et al.,* 1985; Gabay and Kushner, 1999). For WKY rats, as used in our study, Arnold and Meyerson (Arnold and Meyerson, 1990) found a basal AGP concentration of 105 ± 11 $\mu g\ ml^{-1}$.

Upon cannulation, a time-dependent rise in AGP levels was found, with a maximum of 1535 ± 122 $\mu g\ ml^{-1}$ at two days and a gradual return to baseline within seven days post surgery. Since control animals were not anesthetised no distinction can be made between the influence of anesthesia and cannulation in the cannulation(-) group. Several other studies have reported the induction of AGP concentrations following surgery and cannulation. Yasuhara et al. reported a 6-fold increase in AGP levels following a laparotomy in rats two days post surgery (Yasuhara *et al.,* 1985). The increase in AGP concentration at two days post surgery in the present study (Figure 1, cannulation group) is approximately 10-fold compared to control animals. A possible explanation for this observation might be the relative intensity and severity of the surgery or the presence of permanent cannulas in this experiment. The AGP elevation in our experiment returned to baseline within one week without removal of the cannulas and thus does not confirm the results of Terao and Shen in which the α_1-globulin fraction (to which AGP belongs) remained elevated for 15 days post cannulation (Terao and Shen, 1983).

Injection with turpentine oil is one of the most widely used methods to induce local inflammation (Kushner, 1982). AGP serum concentrations have been reported to increase 3- (500 µl s.c.) to even 26-fold (500 µl/100 g s.c.) after turpentine oil administration (Muraikushiya *et al.,* 1993; Jauregizar *et al.,* 2001; Arnold and Meyerson, 1990). In literature, no experiments have been reported describing the effect of turpentine

oil injection in addition to cannulation on protein levels. Our results suggest a more pronounced acute phase reaction in the cannulation(+) group compared to the cannulation(-) group since the maximal AGP response is larger (at 48 hrs) and the levels do not return back to baseline within one week. The variability after cannulation combined with turpentine oil injection was larger and as a consequence the absolute change in the serum AGP concentration did not significantly differ from cannulation alone. Moreover, turpentine oil is irritating to the skin and an injection results in discomfort to the animal. Thus, also from an ethical point of view, cannulation alone is proposed as an animal model for the investigation of the role of serum protein binding in *in vivo* drug effects.

In our studies, the basal levels of RSA (23 ± 1.5, 26 ± 2.5 and 21 ± 0.9 mg ml^{-1}) were found to be smaller than the plasma concentration reported by Murai-Kushiya in control animals (Muraikushiya *et al.,* 1993). The difference might be the result of a difference in rat strain and quantification method.

RSA concentrations are known to decrease upon an acute phase reaction and therefore this protein is called a negative acute phase protein (Ceciliani *et al.,* 2002; Gabay and Kushner, 1999). In the present study, cannulation alone resulted in a decrease in serum RSA concentrations, a 30% reduction post surgery. This change, however, did not reach statistical significance. In the study of Terao and Shen, the RSA concentration decreased 20% from day 2 to day 8 and remained depressed up to 15 days (Terao and Shen, 1983). In their study no comparison was made with baseline values and therefore these results cannot be directly compared with our results.

In contrast, upon cannulation in combination with a s.c. turpentine oil injection our results indicated a significant reduction in the serum RSA concentration form one day (24 hrs) up to four days (96 hrs) post treatment. At two days post treatment, a 38% reduction was observed. This results is in close agreement with literature reports of a decrease in the serum RSA concentration of 29 to 37% two days after a turpentine oil injection (Muraikushiya *et al.,* 1993; Eberini, Agnello *et al.,* 2000; Schreiber *et al.,* 1982). While for AGP a strong interaction between time and treatment effect is observed, this is not seen for the RSA serum levels ($p>0.05$).

The change in serum AGP and albumin concentrations has an important effect on the free concentration of the highly protein bound drug S(-)-propranolol. For that reason, it was investigated how changes in AGP levels would affect the free concentration of this drug. Upon an increase in the serum AGP concentration from 55 to 675 μg ml^{-1}, the free fraction of S(-)-propranolol decreased significantly from 14 ± 0.6 to 1.9 ± 0.3 % (Figure 3). The robust increase in AGP is obviously more important for the S(-)-propranolol binding than the slight decrease in RSA concentration. In theory, the relationship between the reciprocal of the AGP concentration and the free fraction is linear under the assumption of one binding protein and one binding site (Paxton, 1985). The relationship obtained in this study, however is not completely linear. The influence of RSA binding becomes larger at low concentrations of AGP and therefore the free fraction at a concentration of 55 μg ml^{-1} clearly deviates from the linear relationship.

Terao and Shen, 1983 observed a decrease in free fraction from 27% (no treatment) to 7.4% (day 8 post post cannulation). Similar to the present study, a decrease in free fraction is observed upon an acute phase response.

In conclusion, the cannulation alone results in a change in serum AGP concentration, accompanied by an extended range in free fraction of (S)-propanolol between 14 and 2%. Moreover, the elevation of the AGP concentration and return back to baseline within one week, are extremely useful as it enables the study of the influence of a wide range in free fractions within a single rat. Therefore, we consider cannulation alone as a useful approach for the investigation of the role of serum protein binding as a determinant of the PKPD relationship of highly bound drugs in individual rats

6.5 Acknowledgements

The authors gratefully acknowledge the technical assistance of M.C.M. Blom-Roosemalen and S.M. Bos-van Maastricht and the discussions with Dr. F. MacIntyre (Pfizer, research and development). These investigations were financially supported by Pfizer Ltd, Sandwich, UK.

6.6 References

Arnold FJ, Meyerson LR (1990) Radial immunodiffusion assay for rat alpha 1-acid glycoprotein. *Pharmacol. Biochem. Behav.,* 37: 485-491.

Belpaire FM, De Smet F, Chindavijak B, Fraeyman N and Bogaert MG (2002) Effect of turpentine-induced inflammation on the disposition kinetics of propranolol, metoprolol, and antipyrine in the rat. *Fundam. Clin. Pharmacol.,* 3:79-88.

Ceciliani F, Giordano A and Spagnolo V (2002) The systemic reaction during inflammation: The acute-phase proteins. *Protein Pept. Let.,* 9: 211-223.

Chauvelot-Moachon L, Delers F, Pous C, Engler R, Tallet F and Giroud JP (1988). Alpha-1-acid glycoprotein concentrations and protein binding of propranolol in Sprague-Dawley and Dark Agouti rat strains treated by phenobarbital. *J. Pharmacol. Exp.Ther.* 244: 1103-1108.

Cox EH, Kerbusch T, Van Der Graaf PH and Danhof M (1998) Pharmacokinetic-pharmacodynamic modeling of the electroencephalogram effect of synthetic opioids in the rat: correlation with the interaction at the mu-opioid receptor. *J. Pharmacol. Exp.Ther.* 284: 1095-1103.

Day YS and Myszka DG (2002) Characterizing a drug's primary binding site on albumin. *J. Pharm. Sci.,* 92: 333-43.

Derendorf H, Hochhaus G, Mollmann H, Barth J, Krieg M, Tunn S and Mollmann C. (1993) Receptor-based pharmacokinetic-pharmacodynamic analysis of corticosteroids. *J. Clin. Pharmacol.* 33: 115-123.

Drechou A, Perez-Gonzalez N, Biou D, Rouzeau JD, Feger J and Durand G (1989) One-step purification of rat plasma alpha 1-acid glycoprotein by antibody affinity chromatography: application to normal and inflamed rat sera. *J. Chromatogr.,* 489: 273-281.

Eberini I, Agnello D, Miller I, Villa P, Fratelli M, Ghezzi P, Gemeiner M, Chan J, Aebersold R and Gianazza E (2000) Proteins of rat serum V: Adjuvant arthritis and its modulation by nonsteroidal anti-inflammatory drugs. *Electrophoresis* 21: 2170-2179.

Fournier T, Medjoubi N, Porquet D (2000) Alpha-1-acid glycoprotein. *Biochim. Biophys. Acta.*, 1482: 157-171.
Frape DL (1993) Arterio-venous differences of NEFA during exercise. *Equine Vet. J.,* 25: 4-5.

Gabay C and Kushner I (1999) Mechanisms of disease: Acute-phase proteins and other systemic responses to inflammation. *N. Engl. J. Med.,* 340: 448-454.

Harris P, Chlouverakis C, Gloster J and Jones JH (1962) Arterio-venous differences in the composition of plasma free fatty acids in various regions of the body. *Clin. Sci.,* 22: 113-118.

Jauregizar N, Calvo R, Suarez E, Quintana A, Raczka E and Lukas JC (2001) Altered disposition and effect of lerisetron in rats with elevated alpha1-acid glycoprotein levels. *Pharm. Res.,* 18: 838-845.

Kessler MA, Meinitzer A and Wolfbeis OS (1997) Albumin blue 580 fluorescence assay for albumin. *Anal. Biochem.,* 248: 180-182.

Kopecky V, Jr., Ettrich R, Hofbauerova K and Baumruk V (2003) Structure of human alpha1-acid glycoprotein and its high-affinity binding site. *Biochem. Biophys. Res. Commun.,* 300: 41-46.

Kremer JM, Wilting J and Janssen LH (1988) Drug binding to human alpha-1-acid glycoprotein in health and disease. *Pharmacol. Rev.,* 40: 1-47.

Kushner I. (1982). The phenomenon of the acute phase response. *Ann.N.Y.Acad.Sci.* 389, 39-48.

Mandema JW, Sansom LN, Dios-Vieitez MC, Hollander-Jansen M and Danhof M (1991) Pharmacokinetic-pharmacodynamic modeling of the electroencephalographic effects of benzodiazepines. Correlation with receptor binding and anticonvulsant activity. *J. Pharmacol. Exp. Ther.,* 257: 472-478.

Muraikushiya M, Okada S, Kimura T and Hasegawa R (1993) Effects of Turpentine Oil Pretreatment on Beta-Blocker Pharmacokinetic Parameters in Rats. *J. Pharm. Pharmacol.,* 45: 836-838.
Notarianni LJ (1990) Plasma protein binding of drugs in pregnancy and in neonates. Clin. *Pharmacokinet.,* 18: 20-36.

Pardridge WM and Landaw EM (1984) Tracer kinetic model of blood-brain barrier transport of plasma protein-bound ligands. Empiric testing of the free hormone hypothesis. *J. Clin. Invest.,* 74: 745-752.

Paxton JW (1985) Drug binding to plasma proteins. *N. Z. Med. J.,* 98: 245-248.

Piafsky KM (1980) Disease-induced changes in the plasma binding of basic drugs. *Clin. Pharmacokinet.,* 5: 246-262.

Rowland M and Tozer TN (1995) Clinical Pharmacokinetics - Concepts and Applications, 3rd ed. Williams and Wilkins.

Schreiber G, Howlett G, Nagashima M, Millership A, Martin H, Urban J and Kotler L (1982) The Acute Phase Response of Plasma-Protein Synthesis During Experimental Inflammation. *J. Biol. Chem.,* 257: 271-277.

Spanner S, Hall RC and Ansell GB (1976) Arterio-venous differences of choline and choline lipids across the brain of rat and rabbit. *Biochem. J.,* 154: 133-140.

Terao N and Shen DD (1983) Alterations in serum protein binding and pharmacokinetics of l-propranolol in the rat elicited by the presence of an indwelling venous catheter. *J. Pharmacol. Exp. Ther.,* 227: 369-375.

Van Der Graaf PH, Van Schaick EA, Visser SA, De Greef HJ, Ijzerman AP and Danhof M (1999) Mechanism-based pharmacokinetic-pharmacodynamic modeling of antilipolytic effects of adenosine A(1) receptor agonists in rats: prediction of tissue-dependent efficacy in vivo. *J. Pharmacol. Exp. Ther.,* 290, 702-709.

Wagner OF, Nowotny P, Vierhapper H and Waldhausl W (1990) Plasma concentrations of endothelin in man: arterio-venous differences and release during venous stasis. *Eur. J. Clin. Invest.,* 20: 502-505.

Wright JD, Boudinot FD and Ujhelyi MR (1996) Measurement and analysis of unbound drug concentrations. *Clin. Pharmacokinet.,* 30: 445-462.

Yasuhara M, Fujiwara J, Kitade S, Katayama H, Okumura K and Hori R (1985) Effect of altered plasma protein binding on pharmacokinetics and pharmacodynamics of propranolol in rats after surgery: role of alpha-1- acid glycoprotein. *J. Pharmacol. Exp. Ther.,* 235: 513-520.