Establishment and use of surgical rat models for assessment of organ specific *in vivo* clearance

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The thesis is based on the following three papers:

I. Vestergaard B, Agersø H, Lykkesfeldt J. Nephrectomized and hepatectomized animal models as tools in preclinical pharmacokinetics. Basic & Clinical Pharmacology & Toxicology, 2013;113,75-86.

II. Vestergaard B, Appa RS, Lykkesfeldt J, Agersø H. The kidneys play an important role in the clearance of rFVIIa in rats. Thrombosis Research, 2014;133:1124-1129.

III. Vestergaard B, Thygesen P, Kreilgaard M, Fels JJ, Lykkesfeldt J, Agersø H.The kidneys play a central role in the clearance of rhGH in rats. European Journal of Pharmaceutical Sciences, 2016; 86:29-33.

INTRODUCTION

Pharmacokinetics and metabolism is a central part in the development of new drug candidates. Many development programs for new drug entities have been discontinued due to inappropriate pharmacokinetic profiles in man [1]. Development costs increase as the new drug entities move forward from discovery, to non-clinical development and further into phase I, II and III clinical trials [2]. Therefore it would be of great value to have early knowledge of metabolic patterns, clearance pathways and pharmacokinetics to decrease attrition rate during expensive clinical trials.

Knowledge of metabolic patterns and central clearance organs can be used to guide and improve drug design in drug discovery. Designing new drug entities in a way to reduce or abolish certain drug elimination pathways can essentially increase the chance of getting appropriate pharmacokinetic profiles with suitable plasma half-lifes [3]. Clearance and half-life are pivotal pharmacokinetic parameters when dosage regimens and especially dosing intervals are determined. Thus, plasma half-life in patients will have impact on important issues as patient compliance and convenience. It is well known that increased dosage intervals will increase patient compliance and adherence in treatment of chronic diseases [3, 4]. Furthermore knowledge of metabolism and clearance can be used to choose an appropriate animal species for toxicity studies, which will ease the way into the clinical development program [3].

There are several methodologies which can be employed to investigate clearance pathways in vitro and then used to predict in vivo clearance. These predictions from in vitro into in vivo clearance often lack the complexity of whole body clearance as they are focused on specific cell types or subcellular and enzymatic components. The complexity of the body is often characterized by extrahepatic metabolism, active transporter mechanism in primary clearance organs, adaptive physiology or time- or dose-dependent kinetics which in vitro methodologies cannot predict [3]. Studies in intact animals can in some degree be used to assess these complex interplays in clearance, but they are not automatically appropriate for identification of individual clearance processes. This PhD thesis is focused around nephrectomy and hepatectomy models that could be used to shed light on these organ specific clearance patterns as surgical removal of the kidneys or the liver previously have been shown to be useful in investigation of clearance mechanisms of varies compounds [5-14].

Removal of an organ will essentially give information on the clearance going on outside this organ. Thus, comparing the knowledge obtained from this with clearance in an intact pharmacokinetic model would then give information about what extend the organ is contributing in the clearance.

The drawback of these surgical models is the massively invasive nature that disrupts normal physiology and will inevitably lead to systemic changes and eventually death of the animals. This PhD project has been focused around development of surgical rat models for assessment of renal and hepatic clearance by the use of nephrectomy and hepatectomy. Development of such models requires in-depth microsurgical skills as these include very challenging surgical procedures. Furthermore it is a challenge to keep the rats as close to normal physiology as possible when on the same time paying attention to animal welfare. Due to the animal welfare aspect these models should be considered only to be used under continued anaesthesia, which will affect physiology.

Objectives

The primary objective of the PhD project was to establish, validate and use in vivo animal models of organ specific clearance. The project was focused on clearance of protein compounds using in vivo rat models for assessment of renal and hepatic clearance.

The aim of the PhD project was to establish a nephrectomy and a hepatectomy model in rats. Furthermore, the aim was to use these models to investigate renal and hepatic clearance of recombinant human activated factor VII (rFVIIa) and recombinant human growth hormone (rhGH).

The three specific aims of the PhD project were:

Establishment and validation of a nephrectomy model in rats as a tool to investigate relative importance of renal clearance (Paper I and Paper II).

Hypothesis 1A: A nephrectomy model can be kept under anaesthesia and be close to normal physiology during a 7 hours pharmacokinetic study.

Hypothesis 1B: Surgical removal of the kidneys can be used to assess the relative importance of renal clearance.

Investigate renal clearance of rFVIIa and of rhGH (Paper II and Paper III).

Hypothesis 2A: Nephrectomy significantly reduces clearance of both rFVIIa and rhGH.

Establishment and validation of a hepatectomy model in rats as a tool to investigate relative importance of liver clearance (Paper I and unpublished data presented in the thesis).

Hypothesis 3A: A hepatectomy model can be kept under anaesthesia and be close to normal physiology during a 7 hours pharmacokinetic study.

Hypothesis 3B: A hepatectomy model can be used to assess the relative importance of hepatic clearance.

Clearance definition

Pharmacokinetics and clearance are central parts of this PhD thesis as it is focused around renal and hepatic clearance models of proteins. Pharmacokinetics is essentially the movement of a drug in the body and described as the relationship of drug absorption, distribution, metabolism and excretion over time [15]. In this thesis the focus is on drug metabolism and excretion, combined into drug elimination [16]. Elimination of a drug is usually approached as rate constants or half-lives. This approach is not fit to handle the physiological concept of organ specific elimination, which the clearance parameter on the other hand is [15]. Clearance is typically defined as the volume of plasma that is completely cleared of drug per unit of time and mathematically calculated as the dose given divided by the area under the curve (AUC) of the plasma concentration versus time curve [15, 16]. The classic textbook way of looking at clearance separates total body clearance into renal clearance and hepatic clearance [16]. In this way renal clearance becomes the volume of plasma that is completely cleared of drug in the kidneys per unit of time and likewise in the liver for hepatic clearance. Renal clearance has also been described as total amount of unchanged drug excreted in the urine divided by the area under the curve for the plasma concentration versus time curve [16]. This does not hold true for protein drugs as filtered proteins are reabsorbed by the proximal tubule cells and not excreted unchanged [17]. This thesis is focused around clearance of proteins. Thus, the definition of renal clearance as the volume of plasma that is completely cleared of drug by the kidneys per unit of time will be used. Hepatic clearance will be defined as the volume of plasma that is completely cleared of drug by the liver per unit of time. Both for renal and hepatic clearance the definitions will disregard if it is excreted unchanged or metabolized.

KIDNEYS

Anatomy and physiology

The kidneys are two paired abdominal organs with blood flow from the renal artery and the renal vein and the ureters with urine flow to the bladder (figure 1) [18]. The rat kidneys account for approximately 1 % of total body weight and the blood flow to the kidneys is approximately 12 % of cardiac output [19]. The functional unit of the kidney is the nephron and the rat kidney holds approximately 31.000 of these [20]. The nephron consists of the glomerulus, the proximal tubule, loop of Henle, the distal tubule, which drains into a collecting duct (figure 1) [18, 21]. The glomerulus is the first part of the nephron and here the ultrafiltrate of plasma is produced [22]. Glomerular filtration is a process driven by the blood pressure and blood flow in the glomerular blood vessels, which forces fluid and soluble components in the plasma into the Bowman's space, a part of the glomerulus [18, 21, 22]. The fluid including solutes flows into the proximal tubules, where most filtered proteins, peptides and free amino acids along with glucose and other organic solutes are reabsorbed. In general most reabsorption, takes place in the proximal tubules, whereas the loop of Henle, the distal tubules and the collecting ducts are mainly for concentration and acidification mechanisms, and in total around 99 % of all filtered water is reabsorbed [21].

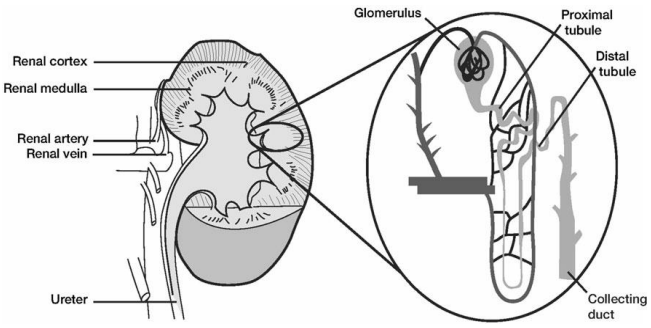

Figure 1

Schematically drawing of renal anatomy. To the left a kidney is drawn with the blood supply shown, as well as the ureter and the division of the kidney into the outer cortex and the inner medulla. To the right a nephron with blood vessels is shown, with the proximal and distal tubule marked. From [18].

Renal clearance

Renal clearance is generally described as the net result of glomerular filtration, active secretion in the proximal tubules, active reabsorption in the proximal tubules and passive reabsorption in the distal tubules [16, 23]. In the case of proteins it has been speculated that renal clearance might also include peritubular receptor mediated uptake from the blood stream followed by intracellular degradation [5].

Glomerular filtration is a process almost like sieving, where plasma can pass through, but blood cells and large proteins cannot [16]. The size plays a very important role in the degree of filtration of a compound, as small compounds pass though very easy, whereas compounds approaching a size of 65 kDa have very limited filtration as seen with albumin, but there is no strict cut off value of size that will result in completely abolished filtration [21, 24]. But the guideline of 65 kDa being some kind of cut off value leads to the general rule that protein bound compounds are not filtered, only the unbound compound is filtered in the glomeruli [16]. Size is not the only thing that matters for the rate of filtration in the kidneys, structure and net charge can also play significant roles [17].

Active secretion in the proximal tubules is a process involving organic anion transporters and organic cation transporters [23, 25]. The classical example of active tubular secretion of a small non-protein molecule is the secretion of penicillin. The organic anion and cation transporters do not transport proteins across the proximal tubule cells and active renal secretion is generally of no significance in renal clearance of proteins. Therefore this is not discussed further.

Passive reabsorption is dependent on a wide range of compound properties including polarity, ionization, molecular weight, water solubility and lipophilicity [16, 23]. Proteins are not reabsorbed by passive reabsorption as they do not have the properties needed for this.

Active tubular reabsorption on the other hand is thought to play an important role in renal handling of proteins. It is well known that multi-ligand transmembrane endocytotic receptors such as megalin and cubilin are found in the proximal tubule cells, and these are part of receptor mediated endocytosis of filtered proteins [26-29]. The uptake of a protein via receptor mediated endocytosis can result in two different fates of the protein. Either it can be recycled back to the blood stream via transcytosis, or it can be degraded in the endozomes and lysozomes of the proximal tubule cells. Active reabsorption of proteins will often lead to endozymatic degradation in the proximal tubule cells [17, 30, 31], which means active reabsorption does not result in reduced renal clearance. The resulting amino acids after endozymatic degradation are then recycled back to the blood stream [26, 32, 33].

In summary, renal clearance of proteins will often be the result of glomerular filtration. It can be speculated that some of the filtered protein is reabsorbed to the circulation via transcytosis in the proximal tubule cells. However most filtered proteins will be endozymatic degradated in the proximal tubule cells.

Renal clearance models

There are several renal clearance models available, but they often have severe drawbacks. Most of the models do not account for the complexity of glomerular filtration with regard to blood flow, blood pressure and filtration rate.

***In vitro* clearance models**

Enzymatic, subcellular and cellular assays are used to assess renal clearance. The obvious drawback of these *in vitro* methods is the lack of glomerular filtration, and renal clearance is often highly dependent on glomerular filtration. Nevertheless there are still studies of receptor-binding in the proximal tubule cells and in immortalized cell lines derived from kidney cells [34]. These can be used to shed light on renal clearance mechanisms rather than a quantitative estimate of renal clearance. Rat kidney slices has been used to address renal clearance of several compounds with minimal metabolism, this has been done with highly varying result, some with good correlations to *in vivo* clearance and others being 100 fold below *in vivo* clearance estimates [35].

***Ex vivo* clearance model**

An *ex vivo* model used to assess renal clearance is the isolated perfused rat kidney model. The model is setup up with perfusion of the kidney via an afferent cannula in either the aorta or the mesenteric artery and an efferent cannula in the vena cava with ligation of all other vessels to the kidney [36]. With both the afferent and the efferent cannula in place it is possible to keep the kidneys perfused and functional for pharmacokinetic studies [36].

Isolated perfused rat kidneys have been used to assess renal filtration for proteins like rhGH [37]. A setup with an *ex vivo* kidney perfusion model will grant information on the degree of filtration, but not renal clearance in the context of total body clearance. Isolated perfused rat kidney models often use a very high flow of the perfusion medium to keep the kidney oxygenated and keeping it functional [36]. Perfusion flow range between 16 to 34 ml/min [38, 39] whereas the normal *in vivo* renal blood flow for one kidney is around 4-5 ml/min [19]. The very high perfusion flow might influence results, however it is unknown to what extend as there is no knowledge about how flow rate will affect glomerular filtration. It has been shown that the isolated perfused rat kidney model can be used to assess active reabsorption [40]. This is potentially of great value as many other models are not able to assess active reabsorption of proteins. In relation to a nephrectomy model the isolated perfused rat kidney lacks the complexity of the whole body interplay between clearance pathways, and the ability to set the measured renal filtration in perspective to total body clearance. But assessment of tubular reabsorption might be possible, which is not possible in the nephrectomy model.

***In vivo* clearance models**

Generally mass balance studies with radiolabelled drug are the classical way for quantitative profiling of parent drugs and metabolites [41]. In these studies it is possible to follow parent drug and metabolites in both urine and feces and thereby quantify the relative importance of the kidneys in the clearance of parent drug. The drawback of mass balance studies is that they very rarely have a recovery of 100 %, and thereby adding error to the estimates [41]. The use of mass balance studies is primarily in studies of disposition of small molecules, rarely for studies of proteins. Which could be explained by the fact that proteins are not excreted unchanged in either urine or feces. Mass balance studies would most likely reflect the clearance of the radioactive isotope itself rather than the parent compound. This is reflected in the current guidelines from the European Medicines Agency (EMA) on disposition of therapeutic proteins [42].

In vivo distribution studies can be used as an indicator of which organs might be involved in the clearance of a protein, and thus relevant for further investigation. Radiolabelled rFVIIa has been used in a several distribution studies showing high concentrations of rFVIIa in the liver and the kidneys [34, 43, 44]. These studies indicate that the primary organs for clearance of rFVIIa would be the liver and the kidneys, but further investigation is needed to confirm this. Here the renal ligation models and nephrectomy models can be used to shed light on renal clearance. These models were reviewed in Paper I.

LIVER

Anatomy and physiology

The gross anatomy of the rat liver is a multilobulated abdominal organ in the cranial part of the abdominal cavity (figure 2) [45]. In the rat, blood flows to the liver via the hepatic artery and the portal vein. Drainage from the liver goes through hepatic veins to the vena cava or straight into vena cava with part of the vena cava being intrahepatic in the rat [45]. The blood flow to the liver in rats is approximately 16 % of cardiac output with about one-sixth of the flow coming from the hepatic artery and five-sixth of the flow coming from the portal vein [19]. The liver produces bile, which flows from the liver run via the bile duct to the intestines. The bile duct exits the liver in parallel to the hepatic artery and

portal vein. Bile is composed of bile acids, cholesterol, phospholipid and inorganic electrolytes on top of that bile is an excretory vehicle of varies metabolites and drugs [46].

The functional unit of the liver is called a lobule (figure 3). The lobules mainly consist of hepatocytes, sinusoidal endothelial cells and kupffer cells [47]. In general the liver has a broad range of functions with great diversity. The spectre of functions vary from amino acid metabolism, plasma protein syntheses and degradation, regulation of lipid metabolism, xenobiotic metabolism as well as uptake and destruction of bacteria, viruses, parasites, and macromolecules, furthermore the liver regulates blood glucose [47]. The liver is central in the regulation of blood glucose; several studies in hepatectomized animal models have demonstrated the need for glucose supplements to keep the animals alive. When reviewing the literature it was clear that several different approaches could be used to control blood glucose. Continuous iv infusion seems to be the best and most used method, but very simple setups with intraperitoneal injections, intragastrical injection, iv bolus injections or subcutaneous injection have been attempted [12, 13, 48-66].

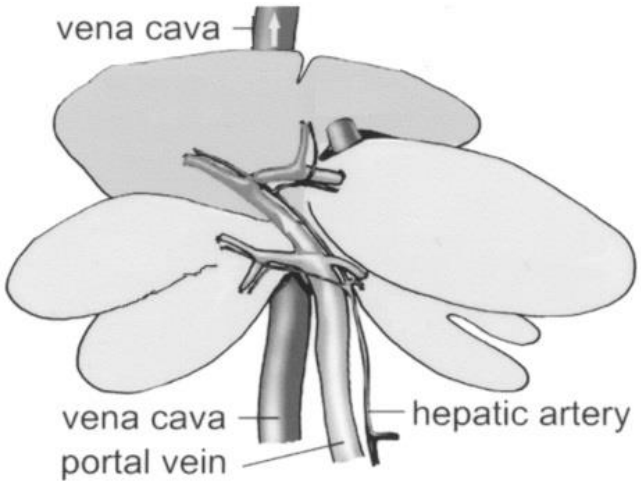

Figure 2
Schematic drawing of the rat liver with main blood vessels. Modified from [67].

Hepatic clearance
Hepatic clearance is comprised of hepatic metabolism and biliary excretion, but it is often reduced to hepatic metabolism as many disregard the importance of biliary excretion. Biliary excretion is an active secretory mechanism, which is considered to be very limited for proteins and therefore also disregarded in the present thesis [16]. Hepatic metabolism is generally considered as being metabolism via cytochrome P450 enzymes (CYPs) [69]. CYPs are known to have many functions such as being part of synthesis of varies endogenous compounds, but also metabolism of a wide range of compounds, especially xenobiotic compounds [69]. Proteins on the other hand are not metabolized via the CYPs. They are metabolized as part of the general amino acid and protein homeostasis in the lysosomes and the proteasomes [70]. Hepatic metabolism and thereby hepatic clearance is highly relevant in the clearance of proteins. There are several well-known endocytotic receptors in liver sinusoidal endothelial cells, hepatocytes and kupffer cells that facilitate hepatic clearance of proteins [71, 72].

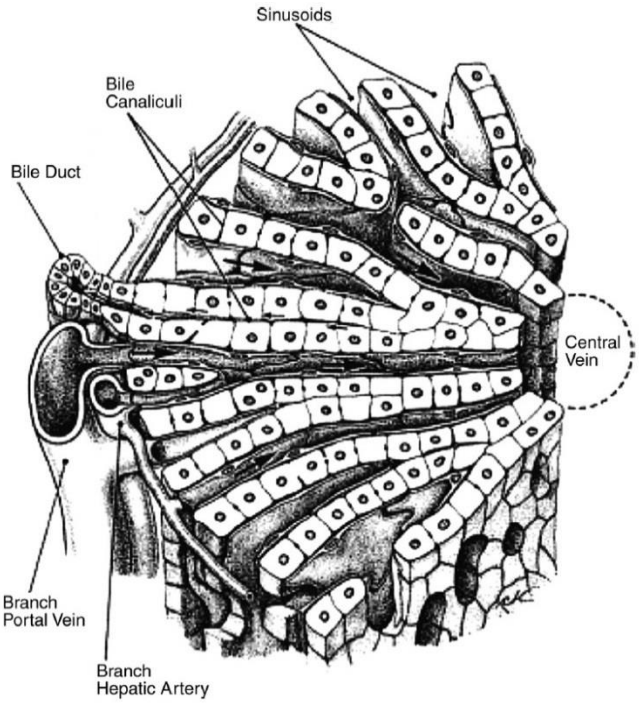

Figure 3
Schematic drawing of the lobular structure of the liver showing the portal triad with a branch of the portal vein, the hepatic artery and the bile duct. Modified from [68].

Hepatic clearance models
Many models can be used to assess hepatic clearance, but they all have drawbacks. Generally it is very challenging to obtain knowledge of the complexity of the body in simplified systems, which will affect the results of hepatic clearance models.

***In vitro* clearance models**
In vitro studies using liver microsomes and isolated rat hepatocytes can be used to predict hepatic clearance and to investigate the intrinsic clearance in the liver [73]. With scaling of the intrinsic clearance it can be translated into hepatic clearance [74]. The drawback of these *in vitro* models is the lack of the whole organ perspective with regard to blood flow and binding to circulating components [74].

***Ex vivo* clearance models**
Isolated rat liver perfusion models have been used to assess hepatic clearance [75, 76]. These models hold great value as the extraction ratio can be determined from these, just as the isolated perfused kidney model was able to assess filtration rate. There are several limitations to the isolated rat liver perfusion models; one major concern is the vitality of isolated hepatic tissue [76]. An isolated perfused rat liver model lacks the complexity of the whole body with respect to the interplay between clearance pathways, nonetheless a liver perfusion model can be used to grant valuable inside into the clearance of proteins [77].

***In vivo* clearance models**
In vivo models used for investigating hepatic clearance could potentially be mass balance studies and distribution studies as described in the section of *in vivo* models for renal clearance. Specific *in vivo* hepatic clearance models can only be obtained with hepatectomy or hepatic devascularization models and these have been reviewed in detail in Paper I.

The perfect surgical anhepatic model would be a hepatectomy model, hence complete removal of all liver mass. This should be done without changing blood flow to both the intestine and the hind part of the body. Creating such a model seems very challenging in rats, as the anatomy of the portal vein and the vena cava hinders this. Removing liver mass will result in changes in the portal blood flow as the portal vein branches into the liver [45]. Changes in portal vein flow can be accommodated in two ways, either by establishment of an anastomosis to the vena cava or by placing some sort of catheter to shunt the blood flow past the liver [6, 78, 79]. The second challenge is the vena cava having a completely intrahepatic part [45], complete removal of the liver requires removal of this part of the vena cava as well. Several methods have been attempted to achieve complete removal of the liver, all of them are very complex. Complete removal of the liver is achieved either by the use of more than one operation with weeks in between or by the use of venous grafts or prosthesis (Paper I, table 4). Venous grafts and prosthesis are surgically very challenging to use and the venous grafts require donor animals.

MODEL COMPOUNDS

The clearance models developed during this PhD project were focused around protein clearance, with rhGH and rFVIIa as model compound of special interest. These two proteins are both recombinant human endogenous proteins, which are marketed as protein drugs used for treatment of chronic conditions. The *in vivo* clearance models developed during this PhD project could also be used for other compounds including non-protein compounds. This will not be described further as it was outside the scope of the project.

The rat was chosen as the species of interest for this project due to general considerations regarding suitable species for *in vivo* experimental work with frequent blood sampling and long term anaesthesia. The laboratory rat fitted the needs and had the perfect size for surgery as well. It allowed for frequent blood sampling unlike mice, where blood volume can be an issue in frequent sampling. The rat is easy to handle single-handedly and often there is very little inter-individual variation in contrast to what is seen in canines and pigs. The rat tolerates long term anaesthesia very well and finally there is a huge amount of historical data from the rat in clearance studies, with the latter being valuable for comparison.

Recombinant activated human Factor VII

Recombinant human activated factor VII (rFVIIa) is the recombinant and activated version of the endogenous human factor VII, a 50 kDa coagulation factor belonging to a family of vitamin K dependent glycoproteins [80]. rFVIIa consists of 406 amino acids and is produced in baby hamster kidney cells [80, 81]. As a product rFVIIa is marketed since 1996 as a haemostatic agent to control bleeding episodes in haemophilia patients with inhibitors against their FVIII or FIX replacement therapy [81, 82].

Recombinant human growth hormone

Recombinant human growth hormone (rhGH) is a peptide hormone produced using recombinant technology, where a strain of *Escherichia coli* is utilized [83]. rhGH consists of 191 amino acids and it is the 22kDa isoform of the human growth hormone [84]. This recombinant product is used for once daily subcutaneous injection for the treatment of pediatric as well as adult patients with growth hormone deficiency, along with several other chronic conditions. rhGH has been on the market since the 1980's [85, 86].

ESTABLISHMENT OF NEPHRECTOMY MODEL

Nephrectomy is surgical removal of the kidneys, often termed bilateral nephrectomy to specify that both kidneys are removed. The nephrectomy model was chosen as the renal clearance model to be used during the PhD project. There are several considerations when setting up surgical models including choice of anaesthesia, maintaining body temperature, monitoring of anaesthesia, the surgical procedure itself, and finally the setup of the clearance study after completed surgery. The nephrectomy model was established using a surgical microscope after making a midline incision. The microscopic approach was chosen to cause the least possible trauma to the rats and to avoid harming the blood flow to the adrenal glands. When looking at other studies using nephrectomized or renal ligated rat models the surgical procedure was performed using an incision in each flank with ligation of the renal blood vessels and removal of the kidneys [5, 87]. This might not be the optimal setup to cause the least possible trauma to the adrenal glands, due to the limited visualisation of the adrenal glands with these small flank incisions. The anatomy of the rat abdomen calls for very gentle and delicate removal of the kidneys to avoid causing trauma to the adrenal glands and their blood supply (figure 3 from Paper I).

Nephrectomy was chosen to avoid the risk of having necrotic tissue, which could potentially increase variation of results or even decreased survival time of the rats. Using a renal ligated model would leave the kidneys inside the abdomen with no blood supply, and thereby eventually result in necrosis of the kidneys. Whether or not necrosis becomes a problem within the 7 hour timeframe of these studies is not clear.

Animals

All procedures were carried out in accordance with "The Danish Animal Experimentation Act" (LBK no 1306 of 23/11/2007). The study was approved by the Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries, Denmark.

In Paper II male Sprague Dawley rats from Taconic, Denmark were used and in Paper III male Sprague Dawley rats from Charles River, Germany, were used. All rats were allowed at least one week of acclimatization. The rats had a body weight of approximately 250 g at the time of the study.

Surgery

Nephrectomy was performed via a midline incision, after which the intestines were wrapped in saline socked gaze and placed outside the abdomen. The renal vein, renal artery and ureter from the right kidney were dissected free and ligated with 4-0 silk suture, followed by removal of the right kidney (figure 4). Afterwards the left kidney was removed in the same way and the intestines were put back in place in the abdomen. The incision was closed by the use of wound clips. Total time, from induction of anaesthesia till completion of surgery and dosing of the rats, was no longer than 30 minutes, and the abdomen was opened for approximately 10 minutes.

Pharmacokinetic setup

After completion of the nephrectomy procedure the rats were subjected to a pharmacokinetic study with iv dosing in a lateral tail vein and blood sampling over time from the opposite lateral tail vein. The lateral tail vein was used because of accessibility, which did not require further surgical intervention. Iv dosing was

performed using a 25G needle and a 1 ml syringe. Blood sampling was likewise done with a 25G needle. Blood was transferred into an Eppendorf tube from the needle.

Blood samples were taken just prior to dosing and at selected time points up to 7 hours post dosing of the nephrectomized rats, resulting in a total time in anaesthesia of approximately 7.5 hours.

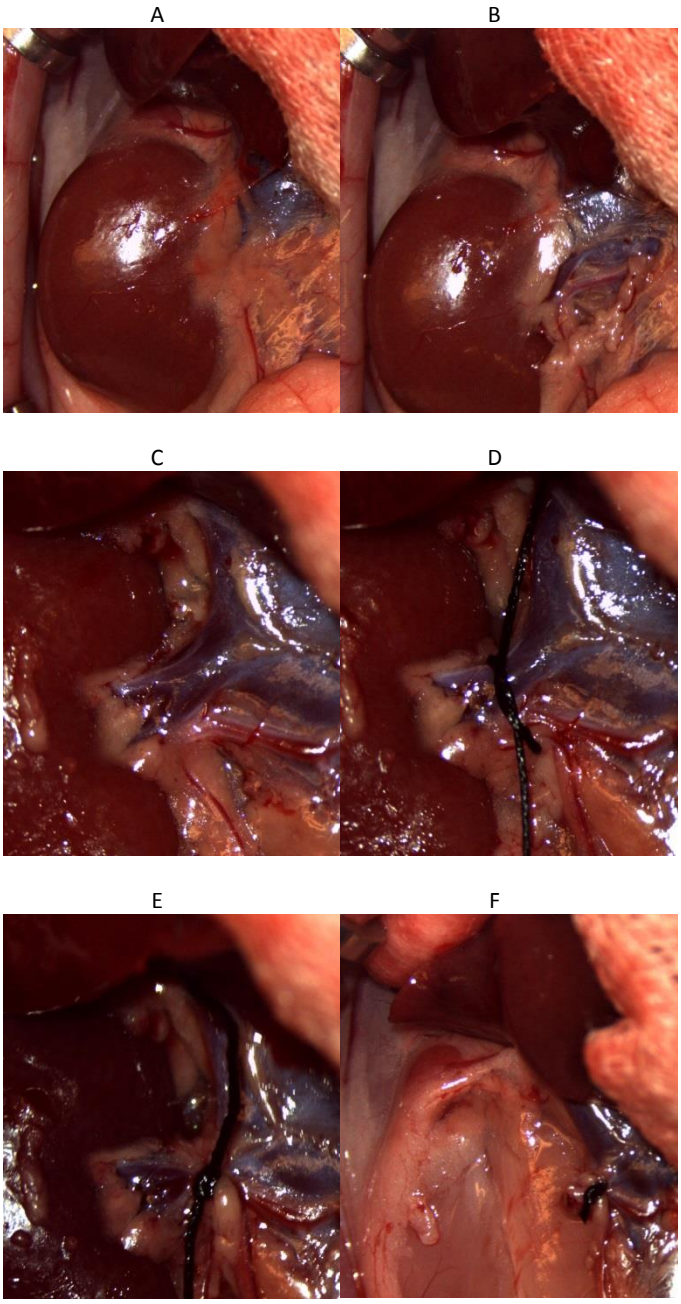

Figure 4
Nephrectomy step by step. A: Exposure of the right kidney trough a midline incision. B: Started dissecting the renal vessel. C: Clear view of the renal artery, renal vein and the ureter after freeing them from adipose tissue by dissection. D: Loosely placed suture around the renal artery, renal vein and the ureter. E: The ligature around the renal artery, renal vein and the ureter has been tightened. F: Completed removal of the right kidney.

Anaesthesia and analgesia
There are several aspects to consider, when choosing anaesthesia for rats undergoing surgery. First and foremost it is important to consider depth and length of anaesthesia. We needed a surgical anaesthesia and length of anaesthesia of up to 7.5 hours. Using injectable anaesthetics would be challenging to control as removal of the kidneys would impact the clearance of injectable anaesthetics, making it very challenging to control anaesthetic depth. Isoflurane was chosen as anaesthetic as it is metabolized in very limited extent, and nephrectomy would therefore have no considerable impact the protocol for anaesthesia. The protocol implemented for anaesthesia was the same for all anaesthetized groups. The rats were dosed with 0.06 mg/kg buprenorphine (Reckitt Benckiser, Berkshire, England) subcutaneously 20 minutes before induction of anaesthesia. Induction of anaesthesia was done in an induction chamber with a flow rate of 0.3 l O_2 and 0.7 l N_2O per minute using 4 % isoflurane (Baxter International Inc, Deerfield, Illinois, US).

Anaesthesia was maintained using a nose cone with a flow rate of 1 l O_2 per minute and 1.5-2.0 % isoflurane.

Monitoring
Rats under anaesthesia were kept on a homeothermic heating blanket system with a rectal probe (Harvard Apparatus). The rectal probe was used for monitoring of rectal temperature and adjustment of the temperature of the heating blanket. It was noted that there was a drop in rectal temperature during surgery even with the highly efficient heating system, but returned to baseline shortly after completed surgery.
Pulse and arterial blood oxygen saturation (SpO2) was monitored while the rats were under anaesthesia. This was done using PulseSense Portable Tabletop Pulse Oximeter (Nonin) with a 2000SL Lingual Clip Sensor (Nonin). The sensor was placed on a paw. Pulse, SpO2 and rectal temperature were recorded preoperatively, at least twice intra-operatively, and every 10 minutes post-operatively until euthanized.

NEPHRECTOMY MODEL RESULTS
A brief summary of the results obtained from the studies using the nephrectomy model will be presented in the following section. These results were presented in detail in Paper II and Paper III. The establishment and validation of the model is presented in Paper II along with rFVIIa clearance and Paper III is focus around rhGH clearance.

Paper II "The kidneys play an important role in the clearance of rFVIIa in rats"
We hypothesized that renal clearance plays a significant role in the clearance of rFVIIa based on *in vivo* distribution studies and studies of proximal tubule cells harvested after dosing of rFVIIa. We further hypothesized that inulin clearance would be almost abolish in nephrectomized rats and renal clearance of both rFVIIa and inulin could be estimated by the use of a nephrectomy model.

In Paper II a total of 24 male Sprague Dawley rats were used and they were divided in groups undergoing no surgery, sham surgery or nephrectomy. The rats underwent a pharmacokinetic experiment with iv dosing of either inulin or rFVIIa, followed by frequent blood sampling post dosing. The pharmacokinetic profiles of inulin and rFVIIa were analyzed by non-compartment analysis as well as non-linear mixed effect modelling using a naïve pooled approach.

The nephrectomy model showed stable rectal temperature, SpO2 and pulse compared to the sham surgery group. This was in line with our aim to keep the model as close to normal physiology as possible.

Inulin clearance was almost completely abolished in the nephrectomized rats whereas the rats that underwent sham surgery had pharmacokinetic profiles similar to those published by others with a terminal half-life around one hour.

rFVIIa clearance was not affected by sham surgery as there was no difference in the clearance between rats only undergoing anaesthesia compared to rats undergoing both sham surgery and anaesthesia. Nephrectomy on the other hand resulted in significant changes in clearance of rFVIIa. Nephrectomy resulted in rFVIIa clearance and terminal half-life of 34 mL/h/kg and 2.8 h compared to 68 mL/h/kg and1.9 h in rats exposed to sham surgery

In conclusion, the nephrectomy model was valid for renal clearance studies. Inulin clearance was almost completely abolished and the data indicate that 50 % of total body clearance of rFVIIa is accounted for by renal clearance.

Paper III "The kidneys play a central role in the clearance of rhGH in rats"

Renal clearance was hypothesized to account for 60-90% of clearance of GH based on previous publications. Thus, the nephrectomy model was hypothesized to be useful to assess the relative importance of the kidneys in the clearance of rhGH in rats.

A total of 24 male Sprague Dawley rats were divided into three experimental groups with eight rats in each, one group was non-anaesthetized, one group was anaesthetized and the last eight rats were anaesthetized and nephrectomized. The rats underwent a pharmacokinetic experiment with iv dosing of rhGH followed by frequent blood sampling until 7 hours post dosing. The pharmacokinetic profiles of rhGH were analyzed by non-linear mixed effect modelling.

The final model that fitted the data of all three groups was a three compartment model with linear clearance and two covariates describing the impact of anaesthesia on clearance and the impact of nephrectomy on clearance. Clearance in non-anaesthetized rats was 290 ml/h/kg, which was reduced to 185 ml/h/kg in anaesthetized rats and even further reduced to 18 ml/h/kg in nephrectomized rats. The difference from non-anaesthetized rats to anaesthetized rats could be caused by the cardiovascular effects of anaesthesia. The change in clearance from anaesthetized rats to the nephrectomized rats was the relative importance of the kidneys in the clearance of rhGH in anaesthetized rats, which was 90 % in this study.

In conclusion, the data from this study in the nephrectomy model indicates that renal clearance accounts for 90 % of total body clearance of rhGH in anaesthetized rats and anaesthesia reduces clearance of rhGH by 36 % in rats.

ESTABLISHMENT OF ANHEPATIC MODEL

There are two ways of achieving an anhepatic model, either surgically or chemically. Chemical anhepatic models can be obtained by administrating hepatotoxic compound such as acetaminophen or galactosamine [88, 89]. Chemical anhepatic models have been used as models of fulminant hepatic failure and were reviewed elsewhere [90]. This PhD project has been focused around surgical procedures. After critical review of the literature we decided to aim for an anhepatic model that could be obtained with one surgical procedure and without the use of venous grafts or prosthesis. The technique used to redirect portal blood flow from the liver to vena cava was modified from a previously described method [78, 91]. This was followed by ligation of the hepatic artery to create a hepatic devascularization model, resulting in a functional anhepatic model.

Animals

All procedures were carried out in accordance with "The Danish Animal Experimentation Act" (LBK no 1306 of 23/11/2007). The study was approved by the Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries, Denmark. Male Sprague Dawley rats from Charles River, Germany were used for establishing the hepatic devascularisation model. All rats were allowed at least one week of acclimatization. The rats had a body weight of approximately 350g at the time of the study.

Surgery

First a midline incision was made and the intestines were wrapped in saline socked gaze and placed outside the abdomen. The surgical procedure was performed using a surgical microscope. The vena cava and the portal vein were dissected free, followed by mobilization of the latter before placing a loose ligature (4-0 silk suture) around the portal vein close to the liver. A purse string suture (6-0 silk suture) was placed in the vena cava at the point where the right renal vein enters the vena cava. A vessel clamp was placed on the celiac artery, the mesenteric arteries as well as on the portal vein. The loose ligature on the portal vein was then tightened and the portal vein was cut. Afterwards it was put trough and flipped over a button and tightened to the button by the use of a 6-0 silk suture. The button used was made from a piece of a teflon tubing [91]. The vena cava was partially clamped by a modified bulldog clamp and an incision was made inside the purse string suture in the vena cava. The button was placed in the opening of the vena cava and the purse string suture tightened. All three clamps were removed and the shunt was inspected for bleeding and patency. Now the portacaval shunt was completed (figure 5). Successful portacaval shunt was confirmed by visual inspection of the colour of the intestines, seeing the venous drainage of the intestines re-established. Afterwards the bile duct and the hepatic artery were ligated and cut followed by closure of the abdominal wound by the use of wound clips. The total surgery time for the hepatic devascularisation model was approximately 1 hour. Completed hepatic devascularisation was assessed by re-establishment of blood flow from the intestine and the need for glucose supplement to keep blood glucose at normoglycemic levels.

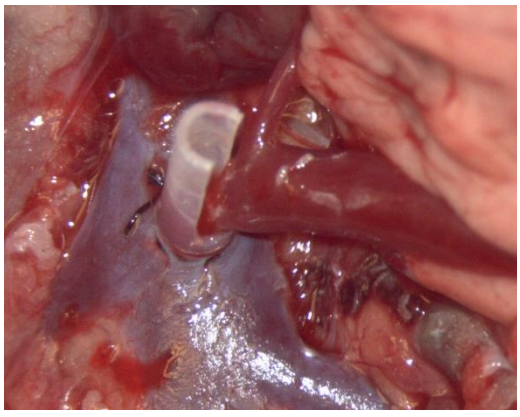

Figure 5
Picture of completed portacaval shunt using the button technique.

Pharmacokinetic setup, anaesthesia and monitoring

The pharmacokinetic setup, anaesthesia and monitoring of the anhepatic rat model was essentially the same as the setup in the nephrectomy model described above. The only difference was

monitoring and regulation of blood glucose in the anhepatic rat model.

Blood glucose

It is essential to keep the anhepatic rat model normoglycemic during a pharmacokinetic study as in any other kind of *in vivo* study. It is especially important in an anhepatic rat model glucose is stored as glycogen and the main glycogen deposits are found in the liver. This results in a compromised blood glucose regulation in the anhepatic state. We tried no glucose supplement, iv bolus injections of glucose as well as iv infusions of glucose to keep the anhepatic rat normoglycemic. Iv bolus injections were done in the lateral tail vein dosing 0.5 ml/kg (50 % glucose solution) every hour. Continuous iv infusions were done with a catheter placed in the jugular vein, inserted just prior to establishment of the anhepatic state. The infusions were done with either isotonic glucose solutions with 5 % glucose or with hypertonic glucose solutions with 50 % glucose. Infusion rates were adjusted according to blood glucose levels, and aimed at reaching a blood glucose level around 8 mmol/l. Blood glucose was measured using Biosen S line glucose analyzer (Eppendorf, Germany). The blood samples were collected from the lateral tail vein in a 5 µl Na-Heparinized capillary tube (Vitrex Medical A/S, Herlev, Denmark) and diluted in 250 µl EBIO buffer (Eppendorf, Germany) prior to analysis.

Evaluation of anhepatic model

Establishment of the hepatic devascularisation model was no trivial task. The model employs a very challenging surgical technique and this resulted in a many failed attempts with massive blood loss from the portal vein, or a non-patent portacaval shunt. The survival time of the hepatic devascularisation model following successfully establishment of the portacaval shunt (figure 5) was significantly correlated with the time the portal vein was occluded ($p = 0.0214$, figure 6). This is in line with what has previously been published, as 15 minutes of total occlusion of the portal vein is enough to cause death, and longer occlusion times lowers the two hours survival rate [92]. Total occlusion without reestablishment of portal blood will result in survival times of approximately 50 minutes [92]. In the present PhD project mean survival time of 213 ± 82 minutes was achieved (n = 17), after successful establishment of the hepatic devascularisation model.

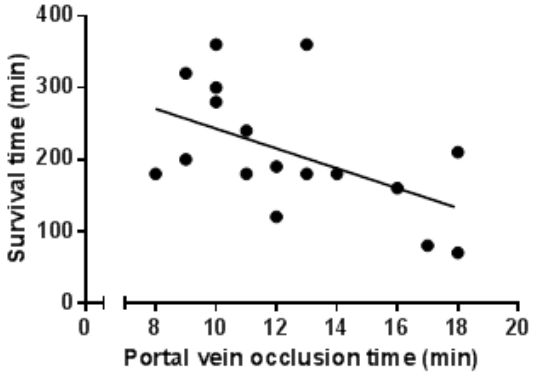

Figure 6

Linear regression of survival time after establishment of the hepatic devascularisation model versus the time of portal vein occlusion during the establishment of the portacaval shunt. Slope -13.82 ± 5.455 and $p = 0.0214$. All rats had iv glucose infusions (n=17).

Optimizing the blood glucose control was part of the development of the hepatic devascularisation model. First it was attempted to keep the animals alive without supplementation with glucose, this resulted in a rapid decline in blood glucose and ultimately death of the rats (figure 7A). The easy way to improve on the blood glucose levels was simply by giving iv bolus injection of glucose, which resulted in very high peak blood glucose levels and only very limited improvement of survival time (figure 7B). Therefore it was decided to use a more advance blood glucose control protocol using continuous iv glucose infusions (figure 7C and 7D). In this optimized setup it was not possible to find significant differences in survival time, but it was clear that the model did seem to be improved with regard to minimum and maximum survival time (table 1).

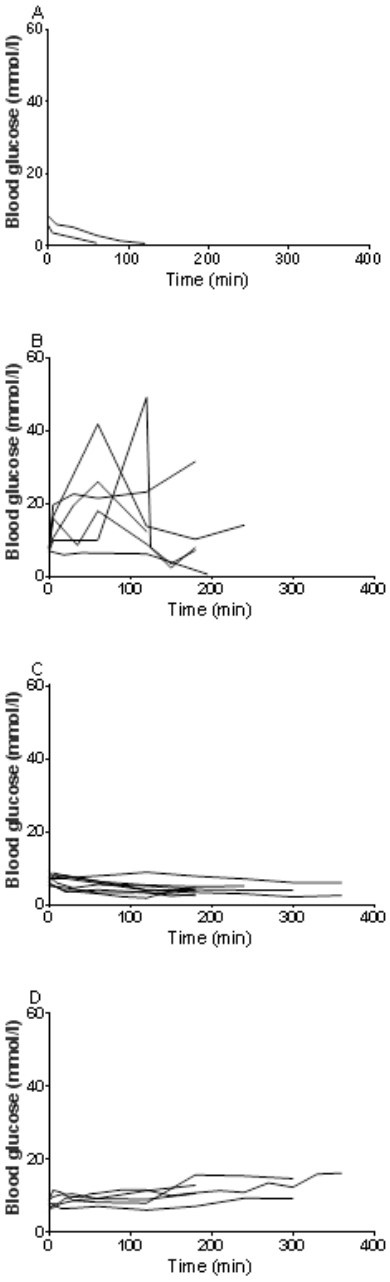

Figure 7

Blood glucose concentration after establishment of the anhepatic state. A: With no glucose supplement, B: Iv bolus injection glucose, C: Iv infusion of isotonic glucose solution, and D: Iv infusion of hypertonic glucose solution.

Table 1

Summary table of survival time after establishment of the hepatic devascularisation model with different blood glucose supplement protocols.

Glucose treatment	No	Bolus	Isotonic infusion	Hypertonic infusion
N	2	7	7	5
Minimum (min)	60	120	180	180
Median (min)		180	180	300
Maximum (min)	150	240	360	360
Mean (min)		182	231	264
SD (min)		35	73	80

The only difference observed between isotonic or hypertonic glucose infusions was a trend in the blood glucose levels. Isotonic glucose infusion resulted in a slow decline in blood glucose levels (figure 8) even with very high infusion flow (mean 5.15 ml/h/kg), whereas the hypertonic glucose infusion had a slow increase in blood glucose (figure 8) with a much lower flow rate (mean 1.5 ml/h/kg).

Rectal temperature, pulse and SpO_2 were measured in rats undergoing hepatic devascularisation (figure 9). The rectal temperature dropped distinctly in the beginning. This drop is most likely caused by a heat loss from the open abdominal cavity during surgery. The temperature stabilized within the first hour post operatively and then it was within normal physiological range for the reminder of the study. This might cause trouble in the assessment of the relative importance of the liver in a clearance study, as whole body metabolism might be reduced with reduced body temperature. Results from measurements of pulse and SpO_2 does not cause any concerns.

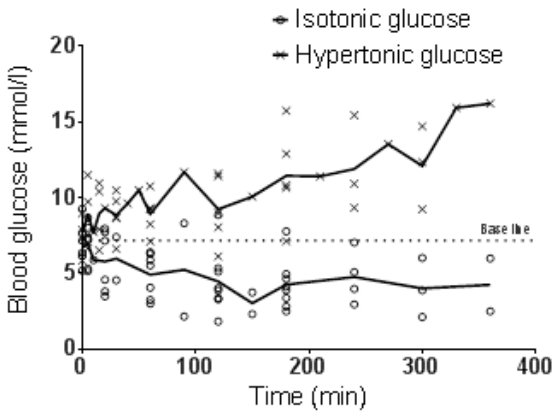

Figure 8
Individual and mean blood glucose concentration versus time after establishment of the anhepatic state with continuous iv infusion of either isotonic or hypertonic glucose solutions. There was no significant difference in survival time between groups.

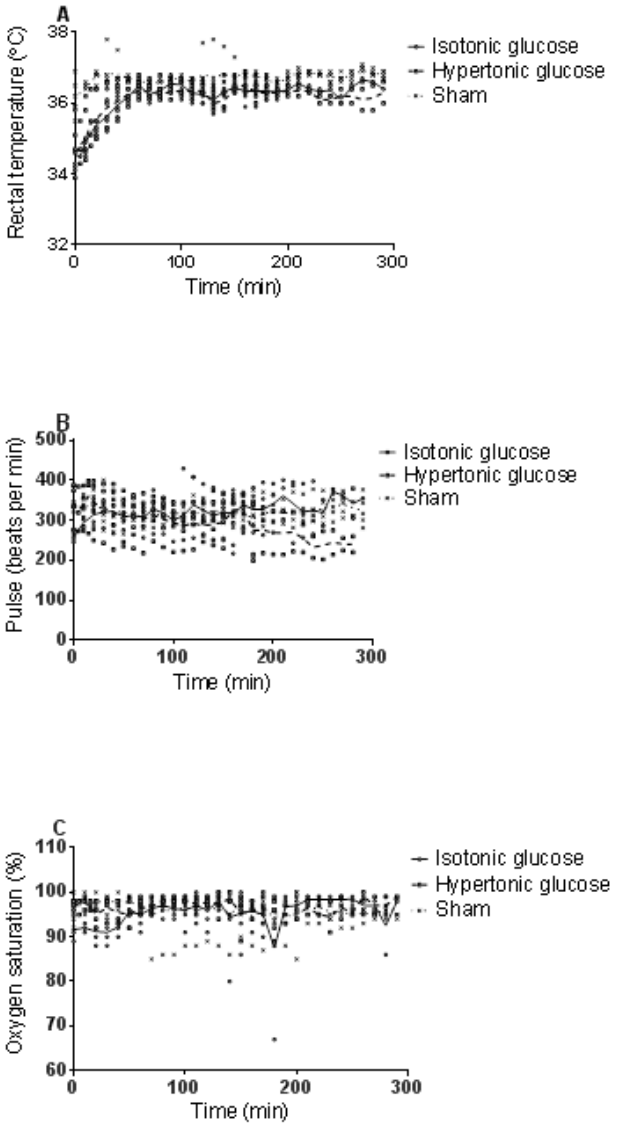

Figure 9
Measures of physiological variables after establishment of the anhepatic state. A: rectal temperature, B: pulse, and C: SpO_2

HEPATIC DEVASCULARISATION MODEL RESULTS

The hepatic devascularisation model was used to assess the relative importance of the liver in the clearance of both rFVIIa and rhGH. The study with rFVIIa was done with isotonic glucose infusion and it was noted that the rats did not survive for the intended 7 hour period of time (figure 10). These data do not support further calculations as they are sparse and of variable quality. Another study was done in the hepatic devascularisation model with hypertonic glucose infusion and looking at the relative importance of the liver in the clearance of rhGH (figure 11). The same pattern was seen with early death of the rats and it was decided not to go any further with the hepatic devascularisation model itself. A non-compartment analysis (NCA) of the rhGH data was performed in spite of the variable quality of the data from the hepatic devascularisation model. NCA of the 8 anaesthetized rats and the 8 nephrectomized rats from Paper III and the 4 hepatic devascularized rats showed that clearance of rhGH in rats

might be explained solely by renal clearance and hepatic clearance (table 2). The sum of the clearance found in nephrectomized rats (49 ml/h/kg) and hepatic devascularized (123 ml/h/kg) add up to the clearance seen in anaesthetized rats (172 ml/h/kg). Nevertheless no significant difference in clearance and AUC was seen between the aneasthetized rats and the hepatic devascularized rats.

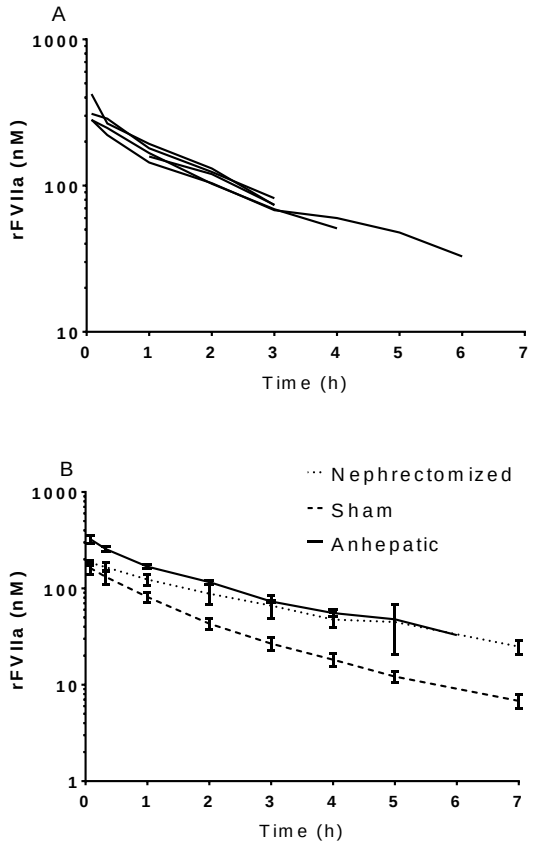

Figure 10
A: Individual pharmacokinetic profiles of rFVIIa in rats after establishment of the anhepatic state. B: Mean plasma concentration versus time profiles of rFVIIa in sham operated rats, nephrectomized rats and anhepatic rats (n = 5 in each group).

Table 2

NCA parameter estimates from anaesthetized (n = 8), nephrectomized (n = 8), and anhepatic (n = 4) rats. $^{*}p < 0.05$, ***: $p < 0.001$ compared to anaesthetized rats.

	Anaesthetized	Nephrectomized	Anhepatic
Cl (ml/h/kg)	172 ± 64	49 ± 9***	123 ± 17
AUC (h*nmol/l)	977 ± 341	3169 ± 591***	1240 ± 164
V_{ss} (ml/kg)	26 ± 15	37 ± 12	64 ± 29*
$t_{½}$ (h)	0.47 ± 0.06	1.01 ± 0.20***	1.00 ± 0.25***
C_{max} (nmol/l)	3349 ± 1243	4900 ± 2336	2730 ± 1485
V_z (ml/kg)	120 ± 54	72 ± 22*	175 ± 39
MRT (h)	0.14 ± 0.03	0.75 ± 0.14***	0.53 ± 0.24***

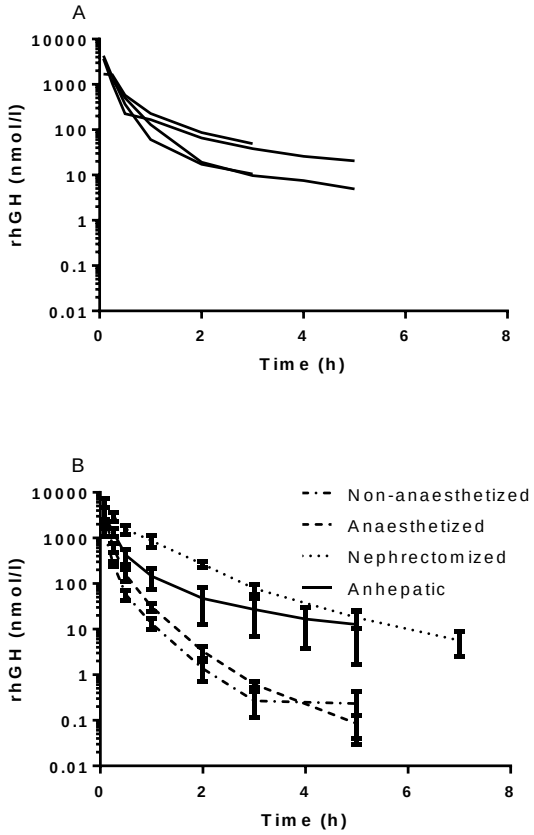

Figure 11
A: Individual pharmacokinetic profiles of rhGH in rats after establishment of the anhepatic state. B: Mean plasma concentration versus time profiles of rhGH in non-anaesthetized, anaesthetized (n = 8), anaesthetized (n = 8), nephrectomized (n = 8), and anhepatic (n = 4) rats.

DISCUSSION

The use of a nephrectomy model and a hepatic devascularisation model in clearance studies was investigated in the present thesis. The overall aim was to establish, validate and use *in vivo* rat models of renal clearance and hepatic clearance. A literature study was conducted to find suitable *in vivo* models and this was written into a review and published as Paper I. A nephrectomy model was successfully established and employed in the investigation of the relative importance of the kidneys in the clearance of both rFVIIa (Paper II) and rhGH (Paper III). A hepatic devascularisation was also established as hepatic devascularisation potentially was easier to establish than a hepatectomy model. The hepatic devascularisation model did not perform as well as expected and thus the model could not successfully be used in the attempt to investigate hepatic clearance of rFVIIa and rhGH.

Use of clearance knowledge

Knowledge of clearance pathways of endogenous proteins such as GH and FVIIa will add knowledge to the further understanding of the function of the given endogenous proteins, and a general understanding of the mechanisms regulating the plasma levels. Recombinant proteins can be used to study the clearance pathways of endogenous proteins. When investigating the physiology of GH, it is clear that some pieces of the puzzle remain unknown.

It is well known that GH has pulsatile plasma concentrations in humans and this is most likely due to the pattern of GH release, but the fast drop in plasma levels of GH from peak pulses is also depend on GH clearance [84].

A major cause of treatment failure of chronic conditions is lack of patient compliance. Compliance with therapy of chronic conditions is known to be affected by dosing interval as it has been shown for the case of osteoporosis patients [4]. There was a significant increase in compliance going from once daily to once weekly and even further increase in once monthly oral dosing. Knowledge of clearance pathways is an important prerequisite for the development of next generation long-acting drug candidates that can improve patient compliance, via an improved pharmacokinetic profile and increased dosing interval.

Physiology

The surgical models used in the present thesis are targeting vital organs and therefore it is to be expected that they have an impact on overall physiology. Due to animal welfare it was decided to keep the rats under continued anaesthesia and analgesia after completion of surgery and this might influence physiological parameters as well. The effects of the surgical procedures and anaesthesia on physiological parameters had to be assessed to validate the models before using them. Removal of an organ will inevitably remove the relevant fraction of total body clearance, but whether this would affect other clearance pathways as well is unknown. Monitoring and control of body temperature was done for both models and it was found that we could keep rectal temperature within normal physiological range during the pharmacokinetic studies using the homeothermic heating blanket [93]. A drop in temperature was noted during surgery, but was normalized shortly after completion of the surgical procedure. The drop in temperature seen during hepatic devascularisation surgery was more profound than the drop seen during nephrectomy. This might be due to the duration of the surgical procedure being. Pulse and SpO_2 was monitored during surgery as well as during the pharmacokinetic studies, no differences were seen between groups. These physiological parameters were used to monitor anaesthesia as well as an indicator of homeostasis. It was decided not to measure blood pressure well knowing that blood pressure is a parameter of relevance. Monitoring of blood pressure would be a very good parameter for the evaluation of homeostasis and it could be speculated that change in blood pressure might also change the metabolic profile of the rat. Measurement of blood pressure is usually done with invasive techniques in rats [94]. The rational for not including blood pressure measurements was focused around not adding further surgical procedures on top of a model, which was already very invasive. As pulse was unaffected by the surgical procedures it was assumed that blood pressure would be unaffected.

Monitoring of blood glucose was needed in the development of the hepatic devascularisation model. The rapid decrease in blood glucose seen in an anhepatic model can result in reduced survival time [53]. In the present studies it was attempted to control blood glucose in different ways. The continuous iv infusions made it possible to keep blood glucose relative constant (figure 7 and figure 8). We saw a rapid decline in blood glucose when not supplementing with glucose. Iv bolus injections of glucose resulted in fluctuating blood glucose levels with very high peak concentrations. Multiple iv bolus injections was challenging and the result obtained was not the stable blood glucose at approximately 8 mmol/l, which we aimed for. The steady increase seen with constant infusion of hypertonic glucose was the preferred way of controlling blood glucose.

Nephrectomy model

A nephrectomy model was developed, validated and used for assessment of renal clearance of rFVIIa and rhGH (Paper II and Paper III). In the process of developing such a model it is essential to review the literature and to learn from the findings described there. In the acute setup used here, we had a model which was approaching normal physiology. This was most likely due to the careful handling of the adrenal glands during surgery, close monitoring and control of rectal temperature and the relative short duration of the study period. Harming the adrenal glands or the blood supply to these could potentially result in changes in physiology as it might affect the release of adrenal gland hormones. E.g. adrenaline, which is released from the adrenal gland to the blood stream and is known to affect blood pressure and heart rate [95]. Changes in adrenaline might therefore result in changed clearance profiles. Body temperature is known to affect metabolism in general, and therefore keeping rectal temperature within normal range was central in the development of the model.

The setup used in this PhD project was an acute model with nephrectomy being performed just prior to the pharmacokinetic setup. The aim was to shorten the time of anaesthesia as well as reducing the time after completed nephrectomy until last blood sample. Time for induction of anaesthesia until collection of the last sample was approximately 7.5 hours. This was done to avoid accumulation of waste product from normal metabolic process. It has been shown that substances present in uraemic blood can affect non-renal clearance [96-98].

The nephrectomy model was tested using inulin, a model compound often used to assess renal function and glomerular filtration [99]. Inulin clearance was almost completely abolished in the nephrectomized rats as seen in figure 3 in Paper II, thus validating that we did not introduce unspecific non-renal clearance of inulin. We therefore concluded that we had successfully achieved establishment of a renal clearance model with physiology being as close to normal as possible.

Following establishment and validation of the nephrectomy model we used it for assessment of renal clearance of rFVIIa (Paper II) and rhGH (Paper III). In Paper II we investigated renal clearance of rFVIIa, and furthermore we investigated whether or not surgery in itself would affect clearance. This was done by introducing a sham surgery group and a group undergoing anaesthesia but no surgery. Sham surgery did not affect the pharmacokinetics of rFVIIa compared to the group only undergoing anaesthesia. This was investigated as surgery could potentially introduce consumption of rFVIIa as an active bleed might use rFVIIa for generating a haemostatic clot. This seemingly was not the case in our model. Comparing clearance of rFVIIa in the nephrectomized rats to the clearance found in the anaesthetized group and the sham surgery group showed that renal clearance accounts for approximately 50 % of total body clearance of rFVIIa in anaesthetized rats. We were the first to quantify the relative importance of the kidneys in the clearance of rFVIIa as well as to quantify renal clearance in itself.

With renal clearance accounting for approximately 50 % it would be highly relevant to investigate a way to reduce renal clearance of rFVIIa, to improve the pharmacokinetic profile. This has been attempted by glycopegylation of rFVIIa with PEG sizes ranging from 5kDa to 40 kDa [100]. Glycopegylated rFVIIa has significant prolonged half-life compared to rFVIIa, which could in

part be explained by reduced glomerular filtration. Glycopegylation had the drawback that the activity of rFVIIa was reduced with increasing size of the PEG, and therefore this might not be the desired way to reduce renal clearance [100].

In Paper III, we investigated renal clearance of rhGH and the effect of anaesthesia on the pharmacokinetics of rhGH. The pharmacokinetic profiles of rhGH in non-anaesthetized rats, anaesthetized rats and nephrectomized rats were modelled simultaneously using population pharmacokinetic modelling. The difference found in the pharmacokinetic profiles could be described by two covariates. One covariate described the impact of anaesthesia on clearance and another covariate described in impact of nephrectomy on clearance. We were the first to use these covariates to quantify the relative importance of both anaesthesia and the kidneys in the clearance of rhGH. There were no other significant differences between groups. Anaesthesia reduced clearance with 36 %. The underlying mechanism of this effect is not completely understood. However, it is well known that isoflurane and buprenorphine affect the cardiovascular system [101-104], which in turn might lead to the changes in clearance observed here. Nephrectomy reduced clearance with 90 % compared to the anaesthetized rats, which is in line with previously published findings pointing towards the kidneys being the most important clearance pathway for rhGH [5, 37, 105]. With knowledge of renal clearance being central in the clearance of rhGH, it would seem logical to attempt to reduce renal clearance when developing new long acting rhGH analouges. Thus, glycopegylation of rhGH has been done and tested in humane subjects [85]. These studies indicate that glycopegylated rhGH could have the pharmacokinetic profile needed for once weekly subcutaneous dosing, but for varies reasons further development of these products have been stopped [85]. The improved pharmacokinetic profile of glycopegylated rhGH could in part be explained by the increase in size resulting in reduced glomerular filtration, which fits well with our findings.

rhGH is used for treatment of patients with end-stage renal disease (ESRD) and here it is of importance to have in-depth knowledge of the clearance mechanisms, especially renal clearance. Indeed a study in ESRD patients has been conducted to investigate the pharmacokinetics and safety of rhGH after sc administration [106]. There were clear differences in the pharmacokinetics of rhGH in ESRD patients compared to healthy subjects, especially with regard to exposure. No safety issues were identified, as the rhGH plasma concentration returned to baseline within 20-22 hours after sc administration, regardless of the investigation being in healthy subjects or ESRD patients [106]. These findings can be explained by the fact that the rate of absorption and the rate of elimination of rhGH after sc administration are relative close to each other in both healthy subjects and ESRD patients [107]. This leads the phenomenon called flip-flop pharmacokinetics with elimination rate being determined by the rate-limiting step in the absorption from the injection site into the blood stream rather than the clearance capacity of the kidneys [108, 109]. Nevertheless flip-flop is only relevant in the case where absorption rate and elimination rate are relative close to each other, if clearance was reduced then the rate-limiting step in elimination might no longer be the absorption rate. Thus, in subjects with normal renal function it would still be desirable to modify rhGH analogues in a way to reduce renal clearance to improve the pharmacokinetic properties, and potentially increase dosing interval when treating patients with GH therapy.

Anhepatic model

One of the objectives of the present PhD project was to establish a hepatectomy model in rats for assessment of hepatic clearance. The surgical procedures used for establishment of anhepatic rat models have previously been described. These were reviewed in Paper I. During the review of the literature it was clear that hepatectomy would involve more profound challenges than hepatic devascularisation. Thus, the anhepatic model chosen for the PhD project ended up being the hepatic devascularisation model. This model was chosen because it could be established with one single surgical procedure, and the level of complexity was lower than hepatectomy. We managed to keep blood glucose levels stable, reduce portal vein occlusion time as well as keeping rectal temperature, pulse and SpO_2 at an acceptable level. Nevertheless the survival time of the model was still relative short and with very high variation (table 1). It could be speculated that the removal of the liver from circulation results in build-up of toxins from normal metabolic process, which will result in intoxication of the rats. This build-up could be highly variable which would then result in variation in survival time. Survival times have been reported to be above 36 hours in one study, indicating that build-up of waste products is not the cause for early death of the anhepatic rat models [65]. If the results of our study were not caused by the removal of the organ from circulation, it might actually be explained by leaving the organ inside the rat after devascularization. In this situation there could potentially be release of varies enzymes and pro-inflammatory cytokines from a necrotizing liver. This has previously been studied by comparing survival times in a three stage hepatectomy model to a three stage hepatic devascularization model in rats [66]. Peignoux and coworkers found that hepatic devascularized rats did not have reduced survival time compared to hepatectomized rats [66]. These results indicate that the necrotic liver after devascularization does not release of any substances that reduce the survival time.

The hepatic devascularization model developed in the present PhD did not meet the required time for good pharmacokinetic studies of rhGH and rFVIIa. The survival time was too short and the variation too high. Nonetheless this model would still hold the potential to investigate hepatic clearance of compounds that would need a shorter time of observation. Minimum survival time of this model was 3 hours when using the hypertonic glucose infusion. Thus up to this study duration the model might be useful, but further validation is needed. It could be relevant to test if inulin clearance would be affected in this model, as this would indicate whether or not extra-hepatic clearance was affected by the surgical procedure. The 3 hour window of observation would be expected to be enough to get good pharmacokinetic profiles for assessment of inulin clearance and thereby further validate the model.

CONCLUSION

The primary objective of the PhD project was to establish, validate and use *in vivo* animal models of organ specific clearance.

The first aim was to establish and validate a nephrectomy model in rats as a tool to investigate the relative importance of renal clearance. This model is now well established and ready to use for investigation of renal clearance of both proteins and non-protein compounds. As hypothesized it was possible to keep the nephrectomy model close to normal physiology during the 7 hours of anaesthesia, and the model could in fact be used to assess the relative importance of the kidneys in clearance of a given compound.

The second aim was to investigate renal clearance of rFVIIa and rhGH in the established nephrectomy model. Renal clearance

and the relative importance of the kidneys were successfully determined for both rFVIIa and rhGH. Thus, for the first time renal clearance and the relative importance of the kidneys in the clearance of rFVIIa was quantified. Likewise, for the first time nonlinear mixed effect modelling was used to quantify the relative importance of both anaesthesia and the kidneys in the clearance of rhGH. Nephrectomy significantly reduced clearance of both rFVIIa and rhGH, which led to the conclusion that the kidneys played a central role in the clearance of these compounds. Renal clearance accounted for 50 % of total body clearance of rFVIIa and 90 % of total body clearance of rhGH.

The third aim was to establish a hepatectomy model in rats to investigate relative importance of liver clearance. Instead of the hepatectomy model, a hepatic devascularisation model was chosen as an anhepatic model for investigation of the relative importance of liver clearance. The conclusion from this study was that the hepatic devascularisation model was in fact an anhepatic model, but it was not possible to keep it under anaesthesia and close to normal physiology for the desired 7 hours after establishment of the anhepatic state. It can be concluded that the anhepatic model was not suitable as a tool to investigate hepatic clearance of the model compounds chosen in this PhD project.

In summary, establishment, validation and use of a nephrectomy model as a tool to investigate renal clearance was successful. But an *in vivo* rat model of hepatic clearance with study duration of the desired 7 hours was not successfully established.

PERSPECTIVES

The nephrectomy model can be used to assess renal clearance of any compound for which pharmacokinetic profiles of around 7 hours or less after iv dosing will grant good estimates of clearance. The model described in this PhD thesis has not been tested beyond 7 hours post dosing. It is expected that the accuracy and validity of the model will decrease with increased time of observation due to accumulation of circulating metabolites, which could affect non-renal clearance.

The hepatic devascularisation model in rats still needs further validation, at the moment it seems unlikely that it will be useful beyond 3 hours post dosing. With further validation it should be possible to assess hepatic clearance of compounds with relative high clearance, which could grant good clearance estimates with frequent blood sampling within the first 3 hours post dosing.

Many drug development strategies are based on optimizing pharmacokinetics properties of already existing drug entities. The clearance models established here can be used to gain valuable insight into clearance mechanisms of a broad range of compounds, and thereby guide which modifications that are warranted to reduce clearance of the given compound in central clearance pathways. The two compounds investigated in this PhD project both have high renal clearance and therefore it would be essential in development of long acting rFVIIa and rhGH analogues to reduce renal clearance. Indeed glycopegylated analogues of rFVIIa and rhGH have been shown to have prolonged circulating half-life, by simply increasing the size of the compound. Increasing size of a compound is a way to reduce glomerular filtration and thereby renal clearance.

These models were developed to assess renal and hepatic clearance of recombinant human proteins, with the aim of gaining new knowledge of the relative importance of the kidneys and the liver in the clearance of these. The value of these models is dependent on the ability to translate the findings from these models to the human situation. The reason for using *in vivo* rat models was to get insight into the complexity of the whole body, rather than using *in vitro* assays, that could potentially use human tissue or cells. Rats are often used in the preclinical setup to investigate pharmacokinetics of potential drug candidates. From the amount of historical data it is possible via various kinds of scaling to get estimates of human pharmacokinetics using these animals. However it is important to bear in mind that there might be other clearance mechanisms in humans than the once found in rats and vice versa. These will not be accounted for using rat *in vivo* clearance models.

In short, our *in vivo* models of organ specific clearance can be used in assessment of the relative importance of a clearance organ, which is an important prerequisite for the development of next generation long-acting drug candidates.

LIST OF ABBREVIATIONS

AUC - area under the curve	iv - intravenous
Cl – clearance	MRT - mean residence time
C_{max} - highest measured concentration	NCA - non-compartment analysis
CYPs - cytochromes P450	rFVIIa - recombinant human activated factor VII
EMA - European Medicines Agency	rhGH - recombinant human growth hormone
ESRD - end-stage renal disease	SpO_2 - arterial blood oxygen saturation
FVIIa - activated coagulation factor VII	$t_{½}$ - terminal half-life
FVIII - coagulation factor VIII	V_{ss} - apparent volume of distribution at equilibrium
FIX - coagulation factor IX	V_z - volume of distribution during terminal phase
GH - growth hormone	

SUMMARY

Knowledge of clearance plays a key role in the development of new drug entities, especially in the development of improved analogous for treatment of chronic conditions. Improved pharmacokinetic properties can be used to increase dosing interval and thereby improve patient compliance. This will lead to improved treatment outcome or decreased risk of treatment failure when treating chronic conditions. Therefore animal models for assessment of organ specific clearance are of great value in preclinical drug development. These models can be used to obtain insights into the relative importance of a clearance organ and thereby guide drug design of new analogues in early drug discovery.

The current PhD project was undertaken to explore surgical *in vivo* models, which could be used in the assessment of the relative importance of major clearance organs. It was the aim of the PhD project to establish and validate both a nephrectomy model and a hepatectomy model as tools to investigate relative importance of renal and hepatic clearance. Furthermore, the project aim was to investigate renal clearance of rFVIIa and rhGH using a nephrectomy model in rats.

The thesis is composed of a short theoretical background, a literature review, two papers based on experimental work as well as experimental work not included in the papers. Chapter one is an introduction with the specific aims and hypotheses. The chapters from two to five contain theoretical background of the clearance concept, anatomical and physiological description of clearance organs and brief overview of potential clearance models including *in vivo* models. Chapters six through nine highlight the experimental work with the results obtained during the PhD project. Lastly, the chapters from ten to twelve contain a general discussion, conclusion and perspectives of the current thesis.

Paper I "*Nephrectomized and Hepatectomized Animal Models as Tools in Preclinical Pharmacokinetics*" provides a literature review of animal models previously used as tools to investigate renal and hepatic clearance. An overview of the surgical procedures previously described for establishment of *in vivo* nephrectomy and hepatectomy models is given. Many different surgical methods have been employed in the attempt to make anephric or anhepatic *in vivo* models. The overall conclusion of the literature review was that a suitable clearance model would require only one surgical procedure. Furthermore, the clearance studies should be conducted immediately after completed surgery to decrease the impact on other clearance pathways and physiology in general.

Paper II "*The kidneys play an important role in the clearance of rFVIIa in rats*" describes the establishment, validation and use of an *in vivo* model for assessment of renal clearance. The model employed was a rat nephrectomy model and the compounds investigated were inulin and rFVIIa. General physiology was assumed to be close to normal as rectal temperature, oxygen saturation and pulse were within normal range during the pharmacokinetic studies. Nephrectomy significantly reduced clearance of rFVIIa and almost completely abolished clearance of inulin. Thus, it was concluded that the nephrectomy model could be used in assessment of the relative importance of the kidneys in the clearance of rFVIIa and the data obtained indicate that renal clearance accounts for 50 % of total body clearance of rFVIIa.

Paper III "*The kidneys play a central role in the clearance of rhGH in rats*" addresses renal clearance of rhGH. The *in vivo* model established in Paper II was used in a pharmacokinetic study of rhGH to assess the relative importance of the kidneys in the clearance of rhGH. The conclusion drawn based on this study was that the kidneys account for 90 % of total body clearance of rhGH in anaesthetized rats. Furthermore, it was noted that anaesthesia reduced clearance of rhGH by 36 % compared to non-anaesthetized rats.

In conclusion, establishment, validation and use of a rat nephrectomy model as a tool to investigate renal clearance was successful, but an *in vivo* rat model of hepatic clearance model was not successfully established.

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