Impact of the Induced Organic anion transporters 1 (Oat1) Renal Expression by Furosemide on the Pharmacokinetics of Organic Anions

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ABSTRACT

Aim Furosemide is a loop diuretic. Different authors demonstrated that continuous administration of furosemide modulates the expression of organic anion transporters. This study was undertaken to simultaneously evaluate the effects of furosemide pretreatment on organic anion transporter 1 (Oat1) and multidrug resistance protein 2 (Mrp2) renal expressions, on p-aminohippurate (PAH) pharmacokinetics and on renal and urinary PAH levels in rats.

Methods Male Wistar rats were treated with furosemide (6 mg/100 g b.w./day, s.c, 4 days) (Treated group) or saline (Control group). On the 5^{th} day, PAH was administered as a bolus infusion in the femoral vein and plasma samples were obtained from femoral artery at different time points. PAH levels in renal tissue and urine were also assessed. Renal Oat1 and Mrp2 expressions were evaluated by Western blotting.

Results Furosemide pretreatment increased both the expression of Oat1 and Mrp2. PAH plasma concentrations decreased following a biexponential function. Furosemide-treated group showed higher PAH plasma levels, a lower systemic clearance and elimination rate constant from the peripheral compartment, indicating that PAH renal elimination was decreased. PAH levels in renal tissue were significantly elevated and in urine appeared to be significantly lower as compared with control animals.

Conclusions Furosemide pretreatment caused a significant decrease of PAH renal elimination, despite Oat1 and Mrp2 augmented renal expression.

The goal of the present study is the addition of important information in the wide gap of knowledge that exists about drug-drug interactions. Due to furosemide worldwide use, the data obtained are interesting and useful in terms of translation to clinical practice.

KEYWORDS

Furosemide, Organic anions, Oat1, Pharmacokinetics, Mrp2

ABBREVIATIONS

PAH, *p*-aminohippurate; Oat1, organic anion transporter 1; Mrp2, multidrug resistance protein 2.

INTRODUCTION

In clinical practice, diuretics are widely used in hypertension treatments and other disorders that are characterized by an excess of extracellular fluid, such as the nephrotic syndrome, chronic kidney disease, cirrhosis and heart failure.[1,2] Loop diuretics such as furosemide, are the most efficient of all diuretics and are highly used and commercialized in many countries. Furosemide inhibits the sodium-potassium-chloride (Na^+-K^+-$2Cl^-$) co-transporter located in the apical membrane of tubular cells in the thick ascending limb in the kidney. [1,3]

Furosemide bounds to plasmatic proteins and is not filtrated by the glomeruli in a large proportion. Therefore, as many other diuretics, needs to be secreted by proximal tubular cells in order to reach its site of action.[2] Furosemide is a weak organic acid, and hence it is secreted by the renal organic anion secretory pathway. The first step in this vectorial transport process is the uptake of furosemide from blood into the cell, which is mainly mediated by the organic anion transporter 1 (Oat1). On that subject, Bartel *et al*.[4] reported that both thiazides and loop diuretics inhibit in vitro tubular uptake of *p*-aminohippurate (PAH, the model organic anion for experimental protocols) in proximal tubule of rabbit kidney. Moreover, Uwai *et al*.[5] described in Oat1-expressing *Xenopus laevis* oocytes, that PAH uptake by this transporter was inhibited in the presence of these two types of diuretics. The second step of this vectorial transport is the efflux of furosemide from the cell into the tubular lumen. Furosemide is primarily eliminated by the kidneys as an unchanged drug but its metabolic clearance via glucuronidation is also remarkable in the kidneys.[6.7] It has been suggested that furosemide efflux from the cell is mainly mediated by the multidrug resistance protein 2 (Mrp2).[7] Bakos et al.[7] using Mrp2-expressing *Spodoptera frugiperda* ovarian cells demonstrated that Mrp2 is responsible for the active secretion of furosemide and other widely used therapeutic drugs.

Oat1 (SLC22A6) is expressed basolaterally in renal proximal tubule and in the choroid plexus. This protein transports a variety of endogenous substrates, such as cyclic nucleotides and urate, products of biotransformation, as well as β-lactam antibiotics, non-steroidal antiinflamatory drugs (NSAIDs), antiviral drugs, and other compounds of pharmacological relevance.[8,9]

Mrp2 (ABCC2) is expressed apically in renal proximal tubule and enterocytes, and in the canalicular membrane of hepatocytes. It is involved in the transport of pravastatin, methotrexate, probenecid, indomethacin, doxorubicin, mycophenolic acid and a wide variety of potentially toxic endo- and xenobiotics in the form of amphiphilic anionic conjugates.[8]

Organic anion tubular secretion is saturable, due to protein transporters are involved in this process, and the administration of different drugs simultaneously can lead to the possibility of clinical drug-drug interactions.[8] Furthermore, alterations in the expression levels of organic anion transporters may have clinical relevance in the elimination of several therapeutic drugs that exist as organic anion compounds at physiological pH that are administered concomitantly with furosemide.[8,9]

So, modulations in basolateral and apical transporters expression and their transport activity through transcriptional and post-translational mechanisms might alter individual pharmacokinetics and drug efficacy.[8,9] In previous studies, different authors have demonstrated that the continuous administration of furosemide modulates the renal expression of organic anion transporters in rats.[10,11]

Both Oat1 and Mrp2 mediate the renal transport of PAH, which is a suitable marker of the renal organic anions secretory pathway. This prototypic organic anion commonly employed in experimental studies undergoes important renal secretion, is scarcely protein bound and

its metabolization is negligible.[12] The basolateral uptake of PAH is mainly mediated by Oat1 as previously reported by Eraly et al.[13] using Oat1 knock out mouse model. On the other hand, Mrp2 has been described as one of PAH transporter in apical membranes from renal proximal tubule cells.[14,15]

In order to foresee possible pharmacokinetic interactions in patients who use furosemide and other anionic drugs at the same time, the aim of this study was to simultaneously evaluate the effects of furosemide pretreatment on Oat1 and Mrp2 renal expressions, on PAH pharmacokinetics and on PAH levels in both kidney and urine in rats.

MATERIALS AND METHODS

Experimental animals

Adult male Wistar rats (aged 110–130 days) were used throughout the study. Animals were cared for in accordance with the principles and guidelines for the care and use of laboratory animals, recommended by the National Academy of Sciences and published by the National Institute of Health [16] and recommended by regulations of the local ethics committee. All experimental procedures were approved by the Faculty of Biochemical and Pharmaceutical Sciences Institutional Animal Care and Use Committee (Res. N°484/2015).

Experimental protocols

The animals were randomly divided into two experimental groups: Control group (Control): rats were treated with furosemide vehicle (600 µL saline/100 g body weight (b.w.)/day, subcutaneously (s.c) for 4 consecutive days; Furosemide group (Treated): rats were treated with furosemide (6 mg/100 g b.w./day, s.c) as previously described, [10,11] for 4

consecutive days. All animals were provided with two separate bottles of drinking water, one containing 0.8% NaCl and 0.1% KCl, and the other containing tap water. On the 4th day, the animals were transferred to metabolic cages for urine collection for the next 18 h, without food to improve urine sample quality as described by Pinches *et al*.[17] The urinary volume (V_U) was determined gravimetrically and was expressed as µL/min/100 g b.w. On the 5th day, all the animals were anesthetized with sodium thiopental (70 mg/kg b.w., i.p.) for pharmacokinetic studies (see below). One set of experimental animals was used for biochemical/physiological parameters evaluation and for kidney plasma membranes preparation (Western blotting studies). Another set of experimental animals was employed for pharmacokinetic studies and PAH levels determinations.

Biochemical determinations

On the day of the experiments, blood samples were obtained by cardiac puncture and blood plasma was separated by centrifugation (1,000 *g* for 10 min). Urine samples were centrifuged at 1,000 *g* for 10 min to remove cell debris. The plasma samples were used for the determination of creatinine $[Cr]_p$ and furosemide levels. The urine samples were used for the determination of creatinine concentrations $[Cr]_u$. Creatinine levels were determined employing a commercial kit (Wiener Laboratory, Rosario, Argentina). Creatinine clearance Cl_{Cr} was calculated employing the conventional formula: $[Cr]_u \times V_U / [Cr]_p$. Furosemide levels were measured using the method described by Bratton-Marshall.[18.]

Pharmacokinetic studies

On the 5th day, animals were anesthetized and a single bolus of PAH (3 mg/100 g b.w., aqueous solution, i.v.) was administered through a femoral venous catheter. Blood samples

from femoral artery were collected at 0, 15, 30, 45 seconds and 1, 2, 5, 15 and 25 minutes after administration of PAH solution. At the end of the experiments, spot urine for PAH analysis was obtained by bladder pressure and kidneys were removed surgically. The animals were euthanized with anesthetic overdose. Blood and urine samples were centrifuged (1,000 *g*, 10 min) and stored at –20 °C until analysis. Concentration of PAH in plasma, urine and renal tissue was measured using the method described by Waugh and Beall.[19]

Kinetic model for the elimination of PAH:

The plasma concentration versus time curves for PAH for each individual animal were fitted with the PKCALC computer program.[20] The choice of the best fit was based on the determination of coefficient values (R^2) and *F* test.[20,21] All fits had R^2 values > 0.9.

Kinetic analysis was done considering a model of two open compartments, because the data fitted as described above were in agreement with this model. PAH concentration decreases in the central compartment following a biexponential function. So, the following equation was used to describe the biexponential concentration–time curves:

$$Cp = Ae^{-\alpha t} + Be^{-\beta t}$$

where Cp is PAH plasma concentration (mg/mL) at time t (seconds or min) after its administration; constants α and β represent the disappearance rates for the distribution and elimination components, respectively, and the slopes of each of the adjusted curves give their values. A and B represent the initial concentrations of the distribution and elimination components, respectively, extrapolated from the y-axis intercept.

Previous kinetic analyses for the elimination of PAH were done in our laboratory using the classical two compartments model. [22,23,24] This model assumes that drug elimination occurs from the central compartment because the major sites of drug elimination (renal

excretion and hepatic drug metabolism) occur in organs, such as the kidney and liver, which are highly perfused with blood. In the present work, we applied the mathematical model originally reported by Richards *et al*.[25] for describing bromosulphthalein pharmacokinetics (an organic anion mainly excreted by the liver). As PAH is an organic anion mainly excreted by the kidneys, it was assumed that kidneys represent the peripheral compartment and consequently drug elimination takes place from the peripheral compartment. To our knowledge, this is the first time that this mathematical model is given to account for the temporal distribution of PAH in blood, kidney and urine, assuming there is no extra-renal loss of this organic anion.

The estimated parameters (α, β, A, B) were used to solve the first-order rate microconstants (k_{12}, k_{21}, k_{20}) with classical equations described by Richards *et al*.[26]. Derived parameters: area under the concentration-time curve (AUC), total volume of distribution (V_{dT}), volume of the central compartment (V_{dC}), volume of the peripheral compartment (V_{dP}), steady-state volume of distribution (V_{dss}), systemic clearance (Cls) and elimination half-life ($t_{1/2}$) were calculated according to standard procedures for compartmental analysis.

Preparation of renal homogenates for PAH determination

The kidneys were removed, decapsulated, cleaned in cold saline, dried and weighed. The renal tissue was minced and homogenized in 30 g/100 mL of ice-cold 250 mM sucrose, 10 mM HEPES-Tris HCl buffer (pH 7.40) and 1 mM PMSF for 5 min at top speed in a *Glas-Col* homogenizer.[13] From this preparation, we obtained total renal homogenates, and aliquots were taken and stored at −80 °C until use.

Preparation of renal plasma membranes for immunoblotting

Renal plasma membranes were obtained according to the method described by Jensen and Berndt [26] as previously reported by us.[11] Aliquots of the membranes were stored immediately at −80 °C for 2 weeks. Each preparation represented renal tissues from four animals. Protein quantification of samples was performed using the method of Sedmak and Grossberg.[27]

Electrophoresis and immunoblotting

Immunoblotting and densitometry for Oat1 and Mrp2 was carried out in renal plasma membranes samples as previously described [11,23,24] using a commercial rabbit polyclonal antibody against rat Oat1 or with a commercial mouse polyclonal antibody against rat Mrp2. Blots were processed for detection using a commercial kit (ECL Plus Western Blotting Detection Reagents; Amersham, Buckinghamshire, UK). A densitometry quantification of the Western blotting signal intensity of membranes was performed using the Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA). To verify equal protein loading and transfer between lanes, Ponceau Red was used as previously described.[11,23,24.]

Materials

Chemicals were purchased from Sigma (St. Louis, MO, USA) and were analytical grade pure. The polyclonal antibody against Oat1 for Western blotting studies was purchased from Alpha Diagnostic International (San Antonio, TX., USA) and the polyclonal antibody against Mrp2 from Abcam (Cambridge, MA, USA). Furosemide was purchased from Sanofi Aventis Laboratory (Argentina).

Statistical analysis

Statistical differences between groups were evaluated using the unpaired Student´s t-test. When variances were not homogeneous, a Welch´s correction was employed. *p* values of less than 0.05 were considered statistically significant. The values are expressed as the means ± standard error (SEM). For these analyses, GraphPad (CA, USA) software was used.

RESULTS

Table I shows a significantly increase of kidney weight and kidney/body weight ratio in Treated animals. Body weight significantly decreased in furosemide-treated animals in comparison with the control group. No modifications were observed in creatinine clearance.

Oat1 and Mrp2 abundance in renal plasma membranes (Fig. 1) were both increased, after the treatment with furosemide.

The pharmacokinetic studies revealed that PAH concentration decreased in the central compartment following a biexponential function (Fig. 2A).

Animals which received furosemide showed a significantly higher area under the curve and therefore a significantly lower systemic clearance for PAH (Fig. 2B) than Control animals. No difference was observed in α, which represents the distribution from the central compartment (Table II). However, animals treated with furosemide showed a lower β compared to Control group, which is an equilibrium constant reflecting the dynamics between k_{21} and k_{20}. In addition, the elimination rate constant from the peripheral

compartment (k_{20}) was significantly lower in Treated group than Control group, indicating an important decrease in this organic anion elimination phase. Total volumes of distribution were not significantly different between groups. A statistically significant decrease was observed in the volume of the central compartment in furosemide-treated animals.

Furosemide plasma levels were measured in order to determine whether the plasma concentration of furosemide may interfere with the excretion of PAH. Plasma furosemide was not detected in both experimental groups.

Net accumulation of PAH in total homogenates of renal tissue was greater in furosemide-treated animals than Control ones (Fig. 3A). Results were expressed as percentage of the administered dose of PAH. On the other hand, a statistically significant decrease was observed in the quantity of PAH excreted in urine of Treated group (Fig. 3B).

DISCUSSION

Renal organic anion transporters are implicated in the pharmacological responses to drugs such as diuretics, and in drug-drug interactions. This is a topic of clinical significance (therapeutic or toxic) Probenecid is a well known inhibitor of renal secretion of other diverse anionic drugs, with the consequent decreasing in drugs excretion.[12] The interaction between methotrexate and NSAIDs has been informed with severe adverse effects after chemotherapeutic treatment. In addition to the direct interaction of drugs with drug carriers, indirect regulation of transporter proteins could be involved in kidney-specific drug interaction.[12] Compounds including drugs, xenobiotics, and endogenous factors, which regulate the expression levels of drug transporters, could influence renal handling of therapeutic drugs being secreted or reabsorbed in the kidney.[8,12]

The frequency of possible drugs interactions increases with the number of simultaneously prescript drugs, and these interactions can produce severe adverse events resulting in harm to the patients.[8,12] Studies of the role of organic anion transporters in drug-drug interaction have been recently initiated, and in the future, the data obtained could be employed for establishing adequate medication for individual patients.

Furosemide is a loop diuretic that is administered along with different drugs in order to regularize hemodynamic function. Oat1 and Mrp2 are two of the main organic anion transporters involved in furosemide tubular secretion [4-7]. In the present work, we observed that furosemide pretreatment increases the protein expression of Oat1 and Mrp2 in a 61 % and 1167 %, respectively. In this connection, Kim *et al.* [10] have demonstrated that prolonged furosemide treatment induces a strong diuresis together with an increase in Oat1 protein abundance in rat kidney. This effect resulted from the furosemide effect itself, since sucrose water loading, which raised urine volume, failed to increase Oat1 renal expression. Moreover, Hazelhoff *et al*.[11] have recently described that a pretreatment with furosemide in rats, increases the uptake of mercuric ions forms into the tubular cells through Oat1 enhanced renal expression and the highest expression of Mrp2 causes an increase in mercuric ions excretion into the tubular lumen thus reducing tubular toxicity due to a decrease in the renal accumulation of mercury.

The Oat1 and Mrp2 up-regulation by long-term administration of diuretics might develop a quicken delivery of active drug to its pharmacological site of action and also to an accelerated renal elimination of different Oat1 and Mrp2 substrates, which will impact in the pharmacokinetics of numerous frequently used therapeutic anionic drugs. The prototype substrate, PAH, has been traditionally used to characterize the organic anion transporting mechanisms. In this work we evaluated if PAH–pharmacokinetics in rats was altered by

furosemide (another organic anion) pretreatment. Furosemide-treated group showed significant higher PAH plasma levels accompanied by a largest area under the curve, a lower systemic clearance and a minor elimination rate constant from the peripheral compartment (k_{20}), indicating that PAH renal elimination was decreased.

PAH accumulation was also assessed in renal tissue and urine. PAH levels in renal tissue were significantly elevated in animals which received furosemide which might be accounted by Oat1 augmented renal expression. On the other hand and consistently with PAH pharmacokinetic parameters described in this work, PAH levels in urine appeared to be significantly lower in furosemide pretreated animals. Taken this together, it would be possible that PAH apical efflux would be impaired, avoiding its urinary excretion and allowing a higher PAH renal tissue accumulation than it was expected from its higher renal inflow mediated by the up-regulation of Oat1 expression. PAH is transported principally by Oat1 in basolateral membranes of proximal tubular cells. After entering the tubular cell, 80 % of PAH is excreted into urine in the intact form and 20 % as an acetylated metabolite (transformed to acetylated PAH in the renal cells).[28] Although PAH uptake by basolateral transporters from blood into the cell has been well characterized, little is known about the apical urinary PAH efflux transporters. In this connection, it has been described that PAH could be excreted into the urine by the Mrp2,[14,15] Mrp4,[29] and BSP/bilirubin binding protein**,** BBBP.[30] In this current work, Mrp2 renal expression was increased the 5th day after furosemide pretreatment. Nevertheless, PAH urinary elimination was decreased. The present data suggest that Mrp2 has not an important role in PAH efflux in vivo, as it was previously demonstrated by Smeets *et al*.[28] These authors showed that PAH excretion in Mrp2-deficient (TR^-) rat kidney was not different from wild-type. On the contrary, mercuric ion urinary excretion in Mrp2-deficient (TR^-) rat kidney was significantly lower,

indicating that Mrp2 plays a role in the physiological handling and renal elimination of mercuric ions, consistently with the results recently described by Hazelhoff *et al*.[11,31]

Nevertheless, it cannot be discarded that accumulated PAH into proximal tubular renal cell (at a high cell concentration accounted by the increased Oat1 expression), could inhibit its own renal efflux by interacting with a secondary binding site that allosterically modulates the apical transporters of this organic anion. In this connection, Zelcer *et al*.[32] proposed that the ATP binding cassette family of transporters has two ligand binding sites which are similar but non identical: one of them from which substrate are transported called S site, and a second one named M site which has the capacity to modulate transport. If a modulator binds to the M site, it induces a structural change that in consequence results in a differential fit of the substrate at the S site.

Beyond the cause that makes PAH accumulation in renal tubular cells in furosemide pretreated rats, the results obtained in this work could be of great relevance because of furosemide treatment might be producing significant alterations in the pharmacokinetics of different drugs that exist as anionic species at physiological pH, decreasing its clearance and increasing its renal accumulation. Nowadays, many patients all around the world are polymedicated. Many of them consumes furosemide, together with other anionic drugs, and therefore particular caution should be taken especially if the latter are also eliminated by renal excretion, and even more, if these drugs are nephrotoxic, because of its possible renal accumulation. Hence, it is of major importance to evaluate substrates interaction with these renal transporters for a better understanding of the cellular pharmacological and toxicological mechanisms of a wide variety of compounds, as well as for the proper planning and adjustment of pharmacotherapy.

The goal of the present study is the addition of important information in the wide gap of knowledge that exists about drug interactions. The data obtained in this work, acquire more relevance if we take into account that furosemide is a therapeutic drug frequently prescribed.

CONCLUSIONS

Furosemide pretreatment causes a decrease of PAH renal elimination in rats in spite of the increased expression levels of Oat1 and Mrp2. PAH accumulation in kidney tissue was also observed. Due to furosemide worldwide use, these results have high pharmacological and toxicological relevancies since the treatment with this diuretic might generate significant alterations in the pharmacokinetics of different organic anion drugs and might produce their renal accumulation. The data obtained are interesting and useful in terms of their potential translation from bench to bedside.

Conflict of Interest

The authors declare that they have no conflict of interest.

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REFERENCES

[1] Reilly RF, Jackson EK. Regulación de la función renal y el volumen vascular. In: Brunton L, Chabner B, Knollman B. (eds). *Goodman & Gilman. Las bases farmacológicas de la terapeútica*, 12nd edn. México DF: McGraw-Hill Interamericana Eds SA, 2012; 671-719.

[2] Sica DA. Diuretic use in renal disease. *Nat. Rev. Nephrol.* 2012; **8**: 100-109.

[3] Oh SW, Han SY. Loop Diuretics in Clinical Practice. *Electrolyte Blood Press.* 2015; **13**: 17-21.

[4] Bartel C, Wirtz C, Brandle E, Greven J. Interaction of thiazide and loop diuretics with the basolateral para-aminohippurate transport system in isolated S2 segments of rabbit kidney proximal tubules. *J. Pharmacol. Exp. Ther.* 1993; **266**: 972-977.

[5] Uwai Y, Saito H, Hashimoto Y, Inui KI. Interaction and transport of thiazide diuretics, loop diuretics, and acetazolamide via rat renal organic anion transporter rOAT1. *J. Pharmacol. Exp. Ther*. 2000; **295**: 261-265.

[6] Kerdpin O, Knights KM, Elliot DJ, Miners JO. In vitro characterisation of human renal and hepatic frusemide glucuronidation and identification of the UDP-glucuronosyltransferase enzymes involved in this pathway. *Biochem. Pharmacol.* 2008; **76**: 249-257.

[7] Bakos E, Evers R, Sinkó E, Váradi A, Borst P, Sarkadi B. Interactions of the human multidrug resistance proteins MRP1 and MRP2 with organic anions. *Mol. Pharmacol.* 2000; **57**:760-768.

[8] Masereeuw R, Russel FGM. Therapeutic implications of renal anionic drug transporters. *Pharmacol. Ther*. 2010; **126**: 200-216.

[9] Wang L, Sweet DH. Renal organic anion transporters (SLC22 Family): expression, regulation, roles in toxicity, and impact on injury and disease. *AAPS J*. 2013; **15**: 53-69.

[10] Kim GH, Na KY, Kim SY *et al.* Up-regulation of organic anion transporter 1 protein is induced by chronic furosemide or hydrochlorothiazide infusion in rat kidney. *Nephrol. Dial. Transplant.* 2003; **18**: 1505-1511.

[11] Hazelhoff MH, Trebucobich MS, Stoyanoff TR, Chevalier AA, Torres AM. Amelioration of mercury nephrotoxicity after pharmacological manipulation of organic anion transporter 1 (Oat1) and multidrug associated resistance protein 2 (Mrp2) with furosemide. *Toxicol. Res*. 2015; **4**: 1324-1332.

[12] Inui K-I, Masuda S, Saito H. Cellular and molecular aspects of drug transport in the kidney. *Kidney Int.* 2000, **28**: 914-958.

[13] Eraly SA, Vallon A, Vaughn DA *et al.* Decrease renal organic anion secretion and plasma accumulation of endogenous organic anions in OAT1 knock-out mice. *J. Biol. Chem.* 2006, **281**: 5072-5083.

[14] Leier I, Hummel-Eisenbeiss J, Cui Y, Keppler D. ATP-dependent para-aminohippurate transport by apical multidrug resistance protein MRP2. *Kidney Int.* 2000; **57**: 1636-1642.

[15] Van Aubel RA, Peters JG, Masereeuw R, Van Os CH, Russel FG. Multidrug resistance protein mrp2 mediates ATP-dependent transport of classic renal organic anion p-aminohippurate. *Am. J. Physiol.* 2000; **279**: F713-717.

[16] Clark JD, Gebhart GF, Gonder JC, Keeling ME, Kohn DF (eds). *Guide for the Care and Use of Laboratory Animals,* Institute of Laboratory Animal Resources. Washington DC: National Academy Press, 1996**.**

[17] Pinches MD, Betts CJ, Bickerton SJ *et al.* Evaluation of novel urinary renal biomarkers with a cisplatin model of kidney injury: effects of collection period. *Toxicol. Pathol.* 2012; **40**: 534-540.

[18] Bratton AC, Marshall EK. A new coupling component for sulfanilamide determination. *J. Biol. Chem.* 1939; **128**: 537-550.

[19] Waugh WH, Beall PT. Simplified measurement of PAH and other arylamines in plasma and urine. *Kidney Int.* 1974; **5**: 429-432.

[20] Shumaker R. PKCALC: a basic interactive computer program for statistical and pharmacokinetic analysis of data. *Drug Metab. Rev.* 1986; **17**: 331-348.

[21] Motulsky H. Using nonlinear regression to fit curves. In: Motulsky H (ed). *Intuitive Biostatistics*. New York: Oxford University Press, 1995; 227-283.

[22] Cerrutti JA, Quaglia NB, Torres AM. Characterization of the mechanisms involved in the gender differences in p-aminohippurate renal elimination in rats. *Can J Physiol Pharmacol.* 2001; **79**: 805-813.

[23] Torres AM, MacLaughlin M, Muller A, Brandoni A, Anzai N, Endou H. Altered renal elimination of organic anions in rats with chronic renal failure. *Biochim. Biophys. Acta* 2005, **1740**: 29-37.

[24] Brandoni A, Anzai N, Kanai Y, Endou H, Torres AM. Renal elimination of p-aminohippurate (PAH) in response to three days of biliary obstruction in the rat. The role of OAT1 and OAT3. *Biochim. Biophys. Acta.* 2006; **1762**: 673-682.

[25] Richards TG, Tindall VR, Young A. A modification of the bromsulphthalein liver function test to predict the dye content of the liver and bile. *Clin. Sci.* **1959**, 18, 499-511.

[26] Jensen RE, Berndt WO. Epinephrine and norepinephrine enhance p-aminohippurate transport into basolateral membrane vesicles. *J. Pharmacol. Exp. Ther.* 1988; **244**: 543-549.

[27] Sedmak JJ, Grossberg SE. A rapid sensitive and versatile assay for protein using Coomassie brilliant blue G250. *Anal. Biochem.* 1977; **79**: 544-552.

[28] Elbourne I, Lumbers ER, Hill KJ. The secretion of organic acids and bases by the ovine fetal kidney. *Exp. Physiol*. 1990; **75**: 211-221.

[29] Smeets PH, van Aubel RA, Wouterse AC, van den Heuvel JJ, Russel FG. Contribution of multidrug resistance protein 2 (MRP2/ABCC2) to the renal excretion of p-aminohippurate (PAH) and identification of MRP4 (ABCC4) as a novel PAH transporter. *J. Am. Soc. Nephrol.* 2004; **15**: 2828-2835.

[30] Torres AM, MacLaughlin M, Quaglia NB, Stremmel W. Role of BSP/bilirubin binding protein on p-aminohippurate transport in rat kidney. *Mol. Cell Biochem.* 2003; **245**: 149-156.

[31] Bridges CC, Joshee L, Zalups EK. MRP2 and the handling of mercuric ions in rats exposed acutely to inorganic and organic species of mercury. *Toxicol. Appl. Pharmacol.* 2011; **251**: 50-58.

[32] Zelcer N, Huisman MT, Reid G *et al.* Evidence for two interacting ligand binding sites in human multidrug resistance protein 2 (ATP Binding Cassette C2). *J. Biol. Chem.* 2003; **278**: 23538-23544.

LEGENDS TO FIGURES

Figure 1. Western blotting for Oat1 (A) and Mrp2 (B) in renal plasma membranes from control (n = 6) and furosemide-treated (n = 6) rats. The abundance of Oat1 in the samples from the experimental animals was calculated as percentage of the mean control value for that gel. The mean of the control value was set as 100 %. Results are expressed as the mean ± SEM. * $p < 0.05$.

Figure 2. Mean plasma concentration–time profiles of *p*-aminohippurate after a single dose (30 mg/kg b.w., i.v.) in control (n = 8, filled circles) and furosemide-treated (n = 7, open circles) rats (A). A comparison of the mean plasma concentration between groups at each time-point was performed. A Student´s t-test showed significant results in the range 0,5-25 minutes. Systemic clearance of *p*-aminohippurate in control and furosemide-treated rats (B). Results are expressed as the mean ± SEM. * $p < 0,05$ vs. control group.

Figure 3. *p*-aminohippurate levels in renal tissue (PAHr) (A) and amount of PAH in urine excreted during 25 min following a single dose of PAH (30 mg/kg b.w., i.v.) (B), in control (n = 8) and in furosemide-treated rats (n = 7). Results are expressed as the mean ± SEM. * $p < 0.05$.

Table I Creatinine clearance, body weight, kidney weight and kidney weight/body weight ratio from control and furosemide treated rats.

	Control rats (*n* = 6)	Treated rats (*n* = 6)
Cl_{Cr} (mL/min per 100 g b.w.)	0,52 ± 0,09	0,43 ± 0,04
Body weight $_O$ (g)	373,2 ± 3,7	374,7 ± 3,5
Body weight (g)	358,5 ± 4,6	342, 5 ± 4,8*
Kidney weight (g)	2,32 ± 0,03	2,69 ± 0,05*
Kidney weight/body weight ($\times 10^{-3}$)	6,47 ± 0,01	7,85 ± 0,14 *

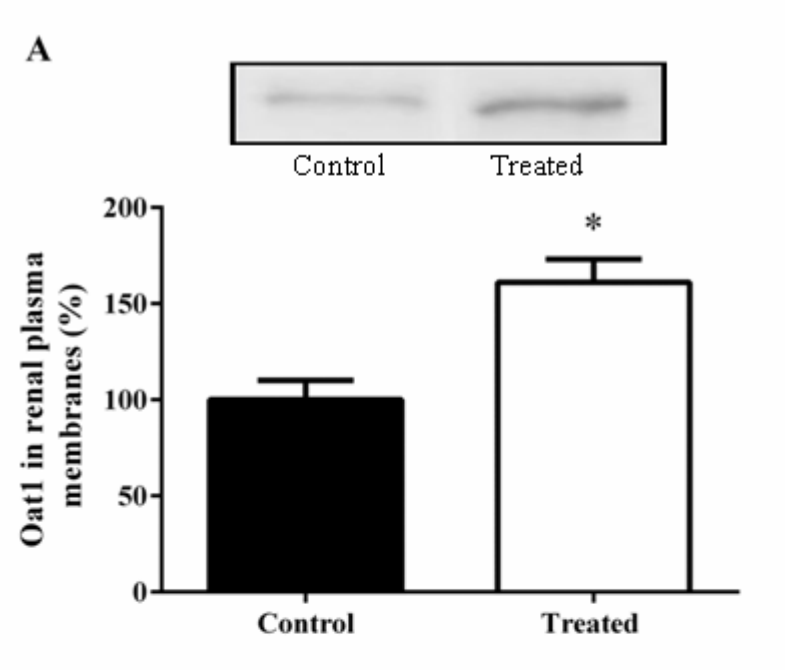
Body weight $_O$ = Initial body weight at time 0, just before the injection of vehicle (control rats) or furosemide (treated animals).
Body weight = Body weight of the day of the experiments
Results are expressed as means values ± SEM * $p < 0.05$.

Table II Pharmacokinetic parameters of *p*-aminohippurate after a single dose (30 mg/kg b.w., i.v.) in control and furosemide-treated rats.

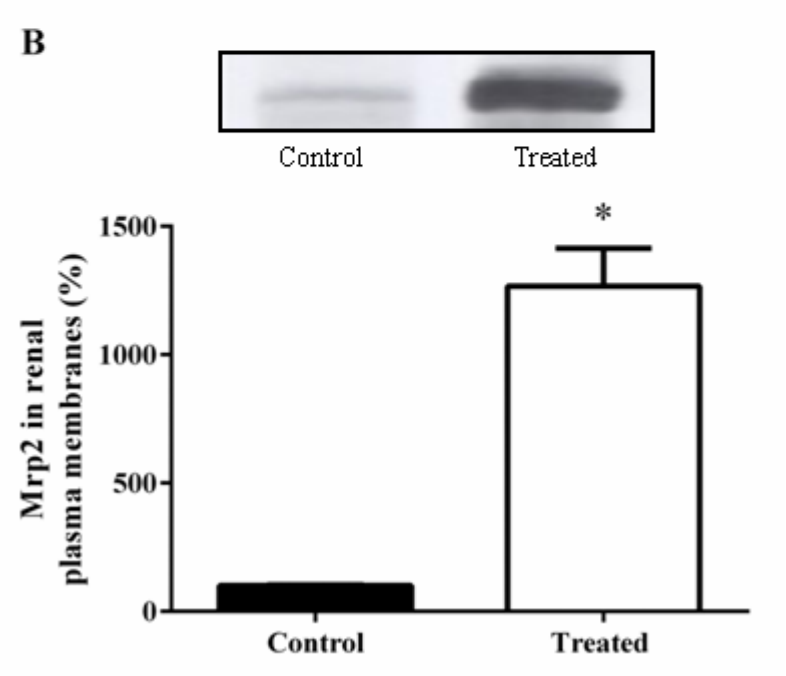
Parameters (units)	Control rats (*n* = 8)	Treated rats (*n* = 7)
AUC (mg/min per mL)	1,46 ± 0,23	4,08 ± 0,83*
Cls (mL/min per 100g b.w.)	2,34 ± 0,27	1,02 ± 0,26*
A (mg/mL)	0,30 ± 0,04	0,40 ± 0,03
B (mg/mL)	0,06 ± 0,01	0,09 ± 0,01*
α (min^{-1})	1,01 ± 0,12	1,18 ± 0,13
β (min^{-1})	0,05 ± 0,01	0,03 ± 0,01*
k_{20} (min^{-1})	0,06 ± 0,01	0,04 ± 0,01*
k_{12} (min^{-1})	0,86 ± 0,11	0,98 ± 0,11
k_{21} (min^{-1})	0,15 ± 0,02	0,19 ± 0,03
$t_{1/2}$ (β) (min)	14,64 ± 1,91	28,83 ± 5,67*
*V*dT (mL per 100g b.w.)	46,06 ± 5,99	31,94 ± 1,98
*V*dC (mL per 100g b.w.)	9,21 ± 1,03	6,29 ± 0,39*
*V*dP (mL per 100g b.w.)	36,86 ± 5,81	25,64 ± 1,99
*V*dss (mL per 100g b.w.)	34,65 ± 3,15	27,79 ± 1,11

Results are expressed as the mean ± SEM. *$p < 0.05$.
AUC=area under curve; Cls=systemic clearance; *A* and *B*=initial values of the distribution and elimination components, respectively; α and β=disappearance rates for the distribution and elimination rate constants, respectively; k_{20}=elimination rate constant from the tissue compartment; k_{12} and k_{21}=first-order rate microconstants of transfer from the central to the tissue compartments; *t1/2*(β)=elimination half-life; *V*dT=total volume of distribution; *V*dC=volume of the central compartment; *V*dP=volume of the peripheral compartment; *V*dss=steady-state volume of distribution

Figure 1



	Control (n = 6)	Treated (n = 6)
Oat1%	100 ± 10	161 ± 12*



	Control (n = 6)	Treated (n = 6)
Mrp2%	100 ± 3	1267 ± 147*

Figure 2

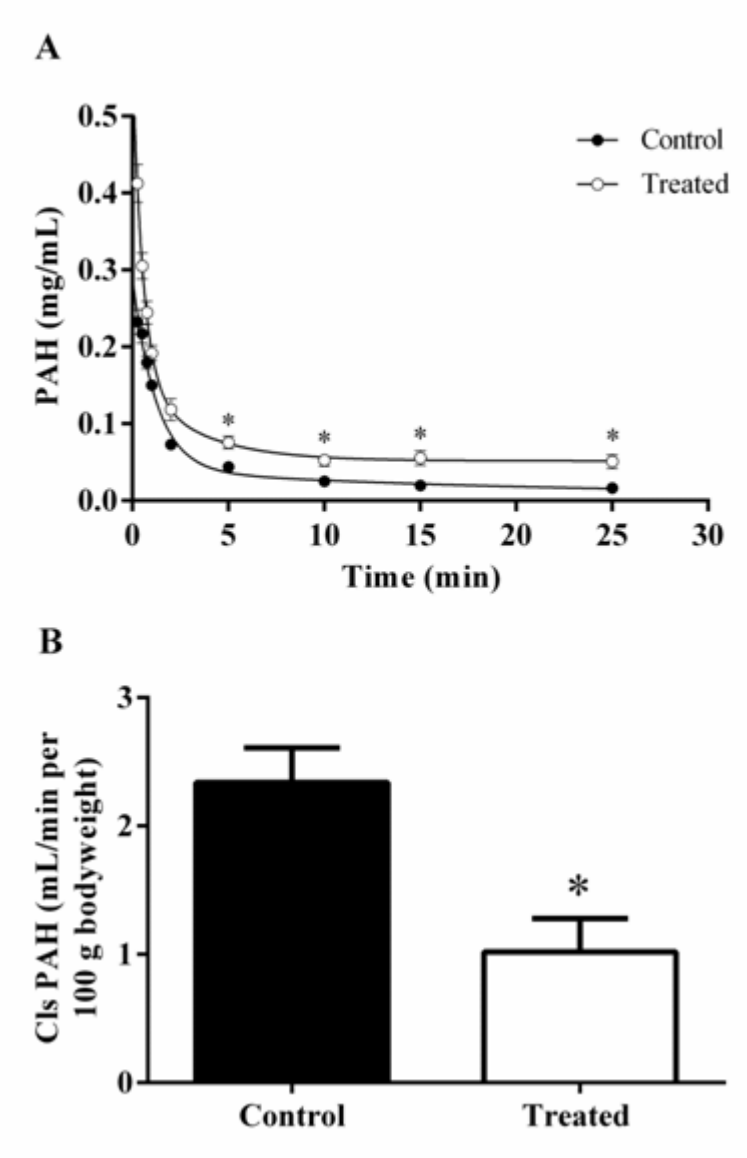
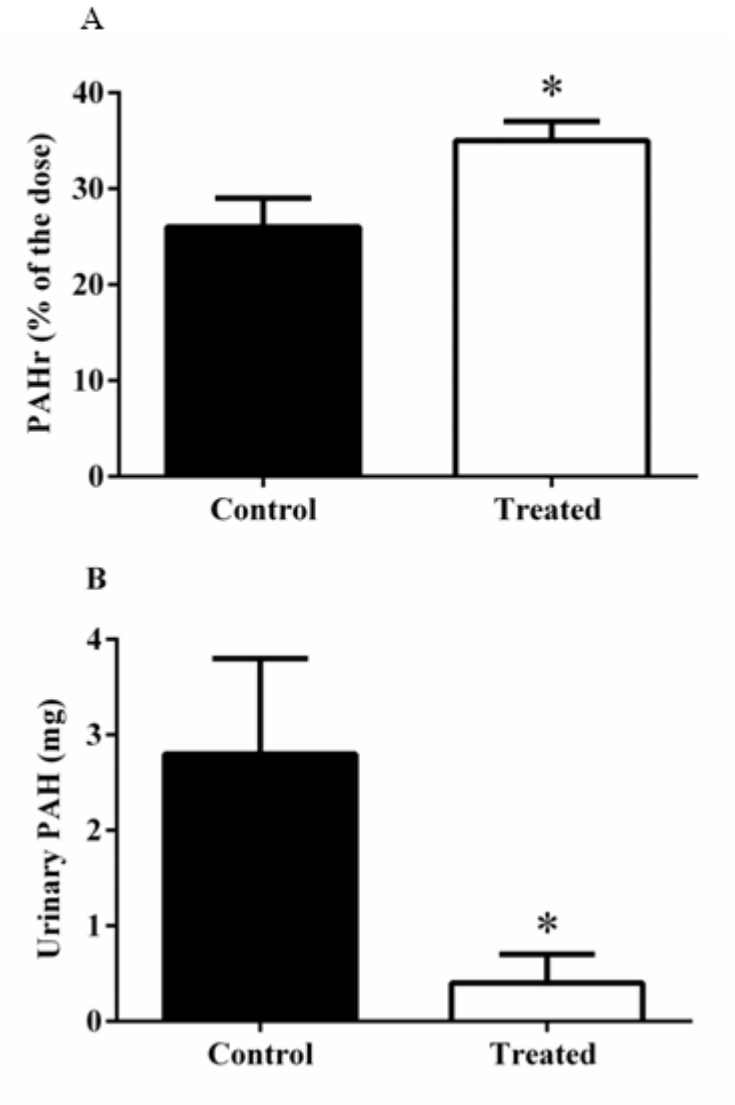

A
0.5
0.4
0.3
0.2
0.1
0.0
PAH (mg/mL)
Control
Treated
*
*
*
*
0
5
10
15
20
25
30
Time (min)
B
3
2
1
0
Cls PAH (mL/min per 100 g bodyweight)
*
Control
Treated

Figure 3


A
PAHr (% of the dose)
40
30
20
10
0
*
Control
Treated
B
Urinary PAH (mg)
4
3
2
1
0
*
Control
Treated