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SPECIAL ARTICLE

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Pharmacokinetics of the antimicrobial drug Sulfanilamide is altered in a preclinical model of vascular calcification

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Summary

In vascular smooth muscle, calcium overload is linked to advancing age. The pharmacokinetics of Sulfanilamide (SA), a compound with antibacterial properties, was evaluated in a preclinical model of vascular calcification. SA was used since it is useful to study possible modifications in the renal and hepatic management of drugs. Vascular calcification was induced by administration of a single high dose of vitamin D_3 to rats (treated group) 10 days before the experiments. A parallel control group was processed. The decrease of renal blood flow due to calcification of the renal arteries explains, at least in part, the decrease in the renal clearance of SA observed in treated rats. The liver metabolic function increased in treated rats as demonstrated by increases in plasma appearance rate of acetylated-Sulfanilamide (ASA), hepatic ASA content and hepatic *N*-acetyltransferase activity. The decrease in renal excretion of SA was not completely compensated by the hepatic metabolism increase, since the elimination rate of SA from the central compartment ($K_{1\text{-}0}$) decreased in the treated group. In summary, in this experimental model with sustained arterial calcinosis induced by a single high dose of vitamin D3 10 days before the experiments, the pharmacokinetics of an aminobenzenesulfonamide is modified, at least in part, by the increase in the activity of hepatic *N*-acetyltransferase and the decrease in renal blood flow. This study emphasizes the importance of considering the presence of vascular calcification when a drug dose scheme is performed, in order to optimize pharmacotherapeutic results.

KEYWORDS
arterial pressure, calcium, hepatic function, *N*-acetyltransferase, pharmacokinetics, renal function, sulfonamides, vascular calcification, vitamin D3

1 | INTRODUCTION

The process of natural ageing of human arteries is reflected in a marked increase in the calcium content. As the age advances, human blood vessels produce a calcium accumulation 5- to 100-fold higher than the respective infantile arteries.[1,2] This is a continuous development process that begins many years before cytotoxic levels of calcium overload are reached. In vascular smooth muscle, calcium overload is a highly pathogenic event that finally leads to calcification and necrotization of the arterial wall. Attempts have been made to study this process and their consequences using available animal models. In these experimental models the vascular overload of calcium associated with advancing age is less pronounced than in humans.[3] Calcium overload with simultaneous structural damage of the arterial walls can be achieved by administration of high doses of vitamin D_3 to young adult rats.[3-12] Under these conditions calcium overload occurs in a few days. In vascular ageing and different vascular human pathologies such as arteriosclerosis, calcium is fixed in the elastic fibres and

makes the vessel more rigid. Vitamin D_3 could accelerate this process in young animals by increasing extracellular calcium levels, modifying the molecular structure of elastic fibres and promoting the synthesis of intermediate proteins that bind calcium ions to elastic fibres.[4] In this experimental model, calcium overload is not limited to the arterial wall and has been observed in many organs such as the kidneys or the heart, which leads to the impairment of the function of the respective organ.[5-12] It is important to mention that the administration of a single high dose of vitamin D3 to young adult rats is essential to obtain the vascular overload of calcium associated with advancing age in animals. As vitamin D3 has a half-life time between 5 and 8 hours,[13] 10 days after its single administration the preclinical model of vascular calcification will be obtained but vitamin D3 level will not be at high levels.

The preserved function of organs, such as the kidneys or liver, is an important determinant of the pharmacokinetics of drugs.[14-16] It is known that impairment of renal function leads to modifications in the renal elimination of drugs mediated by different mechanisms.[14-16] On the other hand, there is a clear interrelationship between the excretory functions of the kidney and liver, so that the damage to one system could be compensated for by the other.[16-19] The integrity of the vascular system is also necessary for a good function of the organs and, consequently, for an adequate management of drugs. Thus, it is possible that injury to the vascular system will allow changes on the pharmacokinetics of drugs.

Although calcium overload is a common event, which progresses with advancing age, there are few reports on drug pharmacokinetics in this experimental model.

The term Sulfonamide (SA) is used as the generic name for derivatives of para-aminobenzenesulfonamide.[20] In the present study this compound, SA, a model compound of sulfonamide group, was used. This is a well known drug, acetylated by the liver and preferentially excreted by the kidneys. SA is a good model to study possible modifications in the renal and hepatic handling of drugs.

We have previously evaluated the pharmacokinetics of SA in a rat model of early arterial calcinosis obtained by a single high dose of vitamin D_3 5 days before the experiment. The results have shown an increase in total body clearance of this drug, probably associated with modifications in its metabolism and/or in organ extraction.[8] Moreover, in the same model, we found modifications in the renal clearance of SA associated with changes in hemodynamics and tubular parameters.[9] Additionally, Quaglia et al.[10] have also demonstrated impairment of renal function in rats with sustained arterial calcinosis induced by a single high dose of vitamin D_3 10 days before the experiment. The increased expression of the organic anion transporters 1 and 3 has recently been reported in the plasma membranes of the proximal renal tubule cells of rats with vascular calcification, which explains the increase in renal clearance of *p*-aminohippurate reported in this preclinical model.[11] Moreover, Hazelhoff et al.[12] have postulated the urinary excretion of the Organic Anion Transporter 5 as a potential noninvasive biomarker of renal injury associated with vascular calcification.

Since renal and vascular system integrity is required for proper drug management, we propose to evaluate the pharmacokinetic parameters of SA and additional parameters that could determine possible modifications of the pharmacokinetics of this drug in rats with sustained vascular calcification induced by administration of a single high dose of vitamin D_3 10 days before the experiments.

2 | RESULTS

The treatment with vitamin D_3 resulted in a large increase in the calcium content of the aortic tissue and an increase in systolic arterial pressure. Plasma calcium levels did not show differences between the two groups studied (Table 1).

2.1 | Pharmacokinetic study

Figure 1A shows the mean plasma concentration-time profiles for SA in control and vitamin D_3-treated rats. The following equation was used to describe the bi-exponential concentration-time curves for SA:

$$C = A e^{-\alpha t} + B e^{-\beta t}$$

TABLE 1 Systolic arterial pressure, total calcium levels in aorta and plasma in control and vitamin D_3-treated rats. Results are expressed as mean±standard error (SE)

	Control (n=9)	Treated (n=7)
Systolic arterial pressure (mmHg)	96±3	136±8*
Total calcium in aorta (μmol/g dry wt.)	23±5	50±5*
Total calcium in plasma (mg/dL)	9.40±0.35	9.70±0.32

*P<.05.

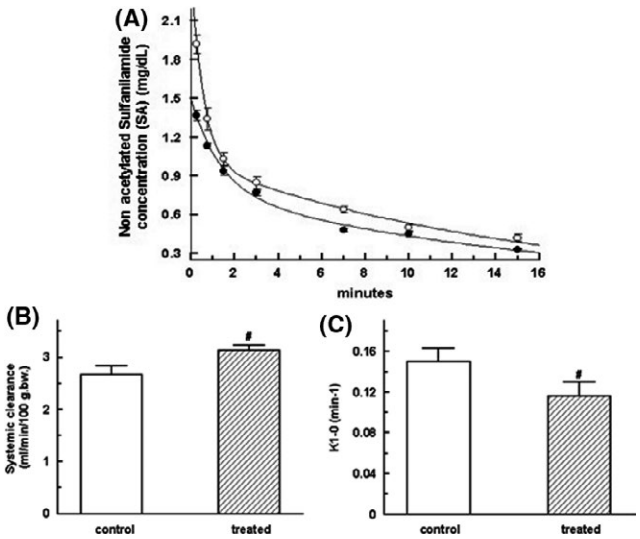

FIGURE 1 (A) Mean plasma concentration-time profile of nonacetylated Sulfanilamide (SA). (B) Systemic clearance of SA and (C) elimination rate microconstant from the central compartment in (○) control (n=10) and (●) vitamin D_3-treated (n=7) rats. Results are expressed as mean±standard error (SE). #P<.05

where *C* is the SA concentration at time *t* after SA administration, α and β are the rate constants for the distribution and elimination phases, respectively. *A* and *B* represent the initial (extrapolated) concentrations of the distribution and the elimination phases, respectively. Plasma SA concentration decreased more rapidly in treated rats.

The estimated parameters (α, β, *A*, *B*) were used to solve the first-order rate microconstants of transfer ($K_{1\text{-}2}$ and $K_{2\text{-}1}$, which are the distribution rate microconstant from the central to the peripheral compartment and the redistribution rate microconstant from the peripheral to the central compartment, respectively) and the elimination rate microconstant from the central compartment ($K_{1\text{-}0}$) with classical equations. The derived parameters: area under the curve (AUC), total volume of distribution (VdT), volume of the central compartment (VdC), volume of the peripheral compartment (VdP), systemic clearance (Cl) and half-life ($t_{1/2}$), were calculated according to standard procedures for compartmental analysis.

Table 2 shows the pharmacokinetic parameters of SA. An increase in the systemic clearance of SA (Figure 1B) and a decrease in the elimination rate microconstant from the central compartment (Figure 1C) were observed in treated rats.

The total compartment volume did not change with the treatment; however there was an increase in the volume of the central compartment and a decrease in the volume of the peripheral compartment in animals with vascular calcification (Table 2).

TABLE 2 Pharmacokinetic parameters of Sulfanilamide after a single dose (4.00 mg/kg b.w., i.v.) in control and vitamin D_3-treated rats. Results are expressed as the mean±standard error (SE)

	Control (n=10)	Treated (n=7)
AUC (mg/min per 100 mL)	16.60±1.20	13.60±0.40
Cl (mL/min per 100 g b.w.)	2.70±0.17	3.10±0.10*
$K_{1\text{-}0}$ (per minute)	0.150±0.010	0.120±0.005*
$K_{1\text{-}2}$ (per minute)	0.80±0.10	0.40±0.07*
$K_{2\text{-}1}$ (per minute)	0.60±0.06	0.70±0.10
Vd T (mL/100 g b.w.)	44±1	45±3
Vd C (mL/100 g b.w.)	18±1	27±1*
Vd P (mL/100 g b.w.)	26±1	18±1*
$t_{1/2}$ (β) (minute)	12.0±1.0	10.0±0.5
A (mg/dL)	1.40±0.08	0.70±0.04*
B (mg/dL)	0.90±0.03	0.90±0.02
α (per minute)	1.60±0.20	1.20±0.20
β (per minute)	0.060±0.002	0.070±0.003

AUC, area under curve; Cl, systemic clearance of Sulfanilamide; $K_{1\text{-}0}$, elimination rate microconstant from the central compartment; $K_{1\text{-}2}$, $K_{2\text{-}1}$ rate microconstants of transfer from central to peripheral compartments and peripheral to central compartments respectively; VdT, total volume of distribution; VdC, volume of the central compartment; VdP, volume of the peripheral compartment $t_{1/2}$ (β), elimination half-life; *A*, *B*, the initial values of the distribution and elimination components, respectively; α, β, the disappearance rates constants for the distribution and elimination components, respectively.
*$P<.05$.

Figure 2A shows concentration-time profiles for acetylated-Sulfanilamide (ASA) in both experimental groups. In order to evaluate the plasma concentration-time curves for ASA, the following equation was used:

$$C = A(1 - e^{-kt})$$

where *C* is the ASA concentration at time *t* after SA administration, *k* is the appearance rate constant of ASA, and *A* represents the maximum (extrapolated) ASA plasma level.

A statistically significant increase in the plasma appearance rate constant of ASA was observed in the treated group (Figure 2B).

In order to clarify these findings, it was decided to evaluate the content of SA and ASA in liver and kidney homogenates.

2.2 | Determination of SA and ASA levels in renal and hepatic homogenates

The content of SA in liver homogenates was significantly lower in rats treated with vitamin D_3 (Figure 3A). In contrast, hepatic ASA content was increased in the same experimental group (Figure 3B).

Figure 3 also shows content of drugs in renal tissue. The SA content in renal homogenates was significantly lower in the treated group (Figure 3C). The acetylated drug content was not significantly different between control and treated rats (Figure 3D).

The liver is the main organ of drug metabolism. It is known that this ability depends on the enzymatic systems and the hepatic blood flow. Thus, we decided to study the hepatic arterial blood flow. As SA is acetylated we also evaluated the activity of *N*-acetyltransferase. These studies were also performed in the kidneys because they are the main site of SA excretion.

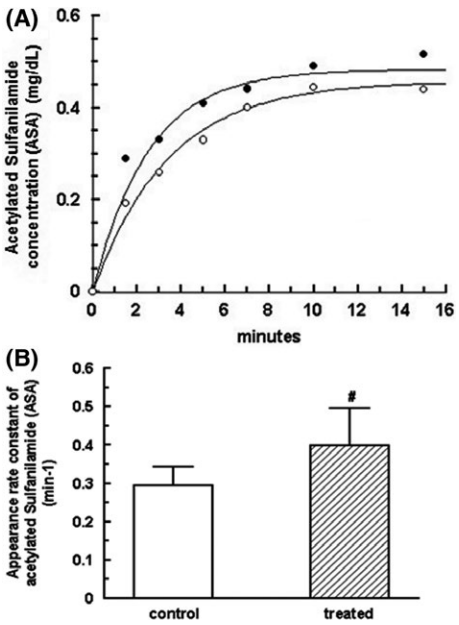

FIGURE 2 (A) Mean plasma concentration-time profile of acetylated-Sulfanilamide (ASA) and (B) plasma appearance rate constant of ASA, in (○) control (n=10) and (●) vitamin D_3-treated rats (n=7). Results are expressed as mean±standard error (SE).
#$P<.05$

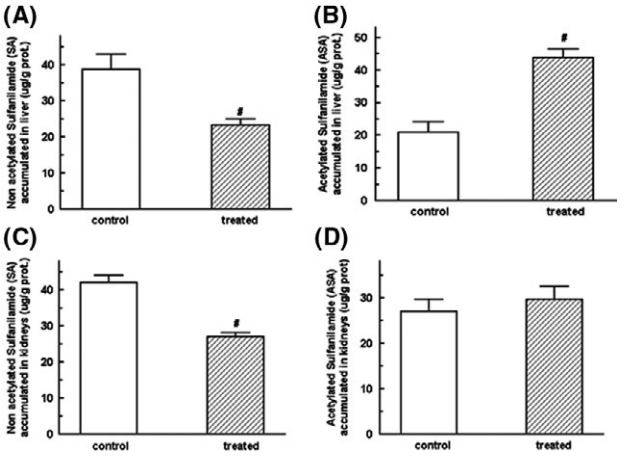

FIGURE 3 (A) Sulfanilamide (SA) and (B) acetylated-Sulfanilamide (ASA) contents in liver homogenates; (C) SA and (D) ASA contents in renal homogenates from control (n=6) and vitamin D_3-treated rats (n=7). Results are expressed as mean±standard error (SE). $^{\#}P<.05$

2.3 | Renal and hepatic blood flow determinations

The hepatic arterial blood flow did not show significant differences between control and treated rats (Figure 4A). In contrast, renal blood flow was significantly lower in rats with vascular calcification (Figure 4B).

2.4 | *N*-acetyltransferase activity assay

N-acetyltransferase activity in liver homogenates was statistically increased in rats treated with vitamin D_3 (Figure 5A). On the other hand, there was no difference between both experimental groups in *N*-acetyltransferase activity in homogenates of kidneys (Figure 5B).

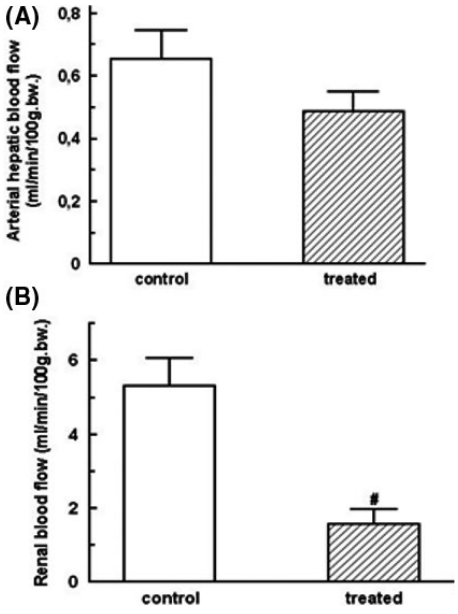

FIGURE 4 (A) Arterial hepatic and (B) renal blood flow in control (n=7) and vitamin D_3-treated rats (n=6). Results are expressed as mean±standard error (SE). (#) $P<.05$

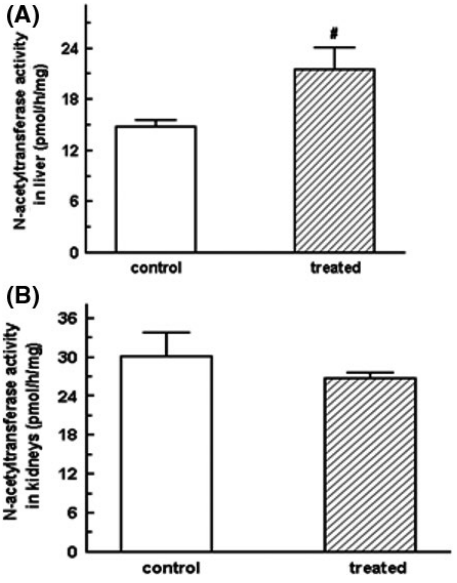

FIGURE 5 *N*-acetyltransferase activity in (A) liver and (B) renal homogenates in control (n=4) and vitamin D_3-treated rats (n=4). Results are expressed as mean±standard error (SE). $^{\#}P<.05$

2.5 | Renal clearance studies

The renal clearance of a drug is often expressed by the concept of a virtual volume of plasma from which the substance is completely eliminated through the kidneys per unit time. The following equation was used to describe the renal clearance (Clr) of SA and ASA:

$$\mathrm{Clr} = C_u \cdot V / C_p$$

where C_u (mg/mL) is SA or ASA concentration in urine, V (mL/min per 100 g b.w.) is urine flow. Finally, C_p (mg/mL) is SA or ASA plasma concentration.

The SA and ASA renal clearance were both significantly lower in treated rats (Figure 6A,B). The study of calcium level in the renal arteries was performed in order to explain, at least in part, some of these results. A significant increase in calcium content in the renal arteries was found in rats with arterial calcinosis (Figure 6C).

3 | DISCUSSION

Ageing induces calcium accumulation in the vascular system. The largest increase is seen in compliance vessels, such as the aorta, but marked increases in small muscular arteries also occur. It is clearly a continuous development process that begins many years before the cytotoxic levels of calcium overload are reached. An increase in calcium content of the arterial wall can be produced in young rats by treatment with vitamin D_3.[1-12]

The aim of the present study was to evaluate the pharmacokinetics of SA in an experimental rat model of vascular calcification induced by a single high dose of vitamin D_3 and to analyze some parameters that could determine possible modifications in the pharmacokinetics of this drug.

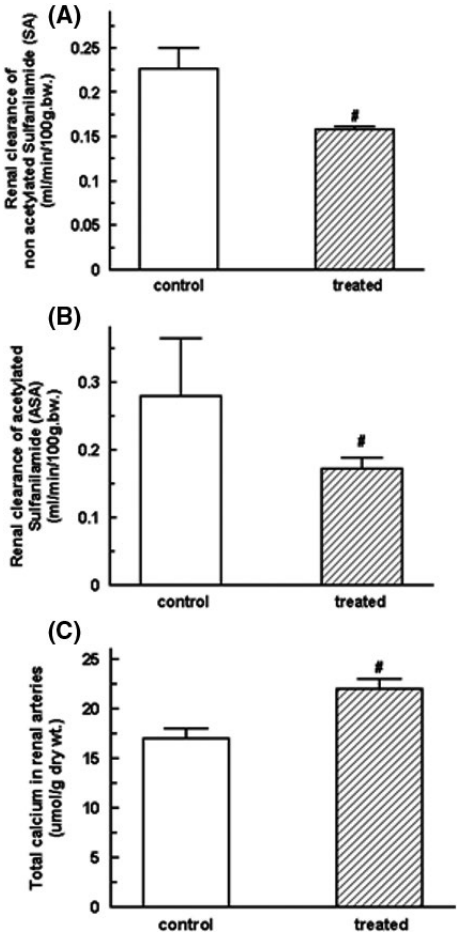

FIGURE 6 Renal clearance of (A) Sulfanilamide (SA) and (B) acetylated-Sulfanilamide (ASA) in control (n=6) and vitamin D_3-treated rats (n=6). (C) Total calcium in renal arteries in control (n=4) and vitamin D_3-treated rats (n=4) Results are expressed as mean±standard error (SE). $^{\#}P<.05$

The rats with vascular calcification showed a large increase in calcium content in the aorta and, in connection with this, an increase in systolic arterial pressure. In this sense, Atkinson[7] found that intracellular free calcium can accumulate in vascular smooth muscle cells and, therefore, alters the contractile properties of these cells. The results of this study corroborate this preclinical model previously described.[5-12]

We have previously shown[8] an increase in systemic clearance of SA in rats with early arterial calcinosis (experimental model obtained by administration of a single high dose of vitamin D_3 5 days before the experiment). In this study, we have evaluated some pharmacokinetic parameters of SA in rats treated with a single high dose of vitamin D_3 10 days before the experiment. These rats showed an increase in the systemic clearance of SA as compared with control rats. The clearance of a drug is generally defined as "a proportionality constant describing the relationship between a substance's rate of transfer, in amount per unit time, and its concentration, in an appropriate reference fluid".[21] Moreover, total body clearance can be expressed as the product of the elimination rate microconstant ($K_{1\text{-}0}$) from the central compartment and the volume of such compartment. As $K_{1\text{-}0}$ decreased in animals with vascular calcification, the increase in the volume of the central compartment observed in these animals could justify the result of the body clearance of SA mentioned above. The increase in the central compartment volume could be due to changes in regional blood flow. In this regard, the administration of 1,25-$(OH)_2$ vitamin D_3 is known to acutely increase the total pressor response in animals infused with catecholamines and may selectively constrict regional circulations.[22] The increased calcium availability within smooth muscle cells would increase arterial resistance.[23] $K_{1\text{-}0}$ is affected by the variables that determine the elimination of drugs from the central compartment, such as the metabolism, renal and biliary excretion. Thus, the decrease of $K_{1\text{-}0}$ observed in treated animals indicates a lower elimination of SA, which could be due to a lower metabolism, a lower excretion or both.

The SA is mainly metabolized by acetylation.[24] Interestingly, this study has shown a higher plasma appearance rate constant of ASA in rats with vascular calcification. In addition, the renal and hepatic content of SA and ASA were evaluated in both control and treated groups. The treated group showed an absolute increase of ASA in hepatic tissue, and there were no variations in renal ASA levels.

N-acetylation is a phase II conjugation reaction mediated by *N*-acetyltransferase. As *N*-acetyltransferase metabolizes SA, we decided to evaluate the *N*-acetyltransferase activity in hepatic and renal tissues. This enzyme is localized in both the liver and the kidneys. The liver is the main site of drug metabolism, which in turn depends on two factors: the metabolic capacity of the liver and the hepatic blood flow.[14] Treated rats did not show modifications in the hepatic arterial blood flow. The increased activity of *N*-acetyltransferase in the liver of rats with vascular calcification may explain the increase of ASA content in the liver and the higher plasma appearance rate of ASA. In this regard, it has been reported that calcium modulates *N*-acetyltransferase activity.[25] Gomez-Cabronero et al.[25] demonstrated that the addition of micromolar levels of calcium to rat spleen microsomes rapidly enhances acetyltransferase activity. This calcium effect was explained by an alteration in the apparent Km of the enzyme for the substrate acetyl-CoA without showing any significant effect in the Vmax of the acetylation reaction. These authors also suggested that calcium modulates acetyltransferase activity by a mechanism that appears to be independent of calmodulin or protein phosphorylation. Moreover, an increase in protein synthesis or a decrease in protein degradation of hepatic *N*-acetyltransferase in this preclinical model of vascular calcification cannot be discarded.

In this study, renal clearance of SA and ASA were also evaluated. These parameters were significantly lower in treated rats. It is well known that impairment of renal function leads to modifications in renal elimination of drugs mediated by alterations in renal blood flow, the glomerular filtration, active tubular secretion and passive tubular reabsorption.[14,15,26] The significant decrease observed in the renal blood flow of treated rats could justify, at least in part, the impairment in the renal elimination of Sulfanilamide. The higher calcium content in the renal arteries observed in treated rats could explain the decrease of renal blood flow. The renal arteries may exhibit increased reactivity to noradrenaline associated with modifications in calcium content. In this regard, acute administration of vitamin D_3 has been reported to produce increased vessel reactivity to catecholamines.[27] In addition,

it was also reported that noradrenaline induces higher levels of intracellular calcium.[28] In this regard, we have described damage in renal function and structure linked to vascular calcification.[9-12] Renal histological studies showed tubular alteration with vacuoles in the cytoplasm and glomeruli of reduced size in rats with vascular calcium overload.[11,12] Modifications were also observed in the hemodynamic parameters and in the tubular parameters that were associated with changes in the activity of the medullar sodium pump and content of medullar calcium.[10] Quaglia et al.[10] have also described a decrease in the glomerular filtration rate (40%), which could also contribute to decreased renal excretion of SA.

In summary, the decrease observed in the elimination rate microconstant ($K_{1\text{-}0}$) of SA from the central compartment in rats with vascular calcification is due to an important decrease in its renal excretion, which is not completely compensated by the increase in its metabolism.

The elimination of drugs from the body is, fundamentally, a concerted action of the liver and the kidneys. Thus, impaired renal function may be associated with an increase in hepatic activity in rats with vascular calcification. In this regard, the reciprocal compensation of renal and hepatic function, concerning drug elimination, has previously been described in the presence of different pathologies.[16-19]

This study highlights the numerous factors that influence the systemic clearance of a drug in the presence of a pathological condition such as vascular calcification, linked to ageing. In this case, alterations in renal excretion and in hepatic metabolism modify the elimination rate from the central compartment of the drug studied. The concomitant modification in this elimination rate with the increase in the distribution volume of the central compartment determines the alteration observed in the systemic clearance of the substance studied in this work.

The data obtained are interesting and useful in terms of translation to clinical/medical practice. The present study remarks the importance of considering the presence of vascular calcification, which is common in ageing process, when a drug dose scheme is performed in order to ensure beneficial pharmacological effects and to avoid toxicological effects.

4 | METHODS

4.1 | Experimental animals and treatment

Adult male Wistar rats (320-380 g body weight) were randomly divided into two groups: control and treated rats. Treated rats were injected with a single high dose of vitamin D_3 (300 000 IU/kg b.w., intramuscular) 10 days before the experiment in order to induce a vascular calcium overload as previously described.[6-12] Vitamin D_3 solutions were prepared in corn oil. The control animals received an identical volume of corn oil by i.m. injection. All animals were housed in rooms with constant temperature (21-23°C) and regular light/dark cycles (LD 12:12 hours). The rats were allowed free access to a standard diet and tap water. The rats 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. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Faculty of Biochemical and Pharmaceutical Sciences (UNR).

4.2 | Experimental procedures

4.2.1 | Measurement of arterial pressure

Systolic arterial pressure was measured in all experimental groups using a Harvard indirect rat tail blood pressure monitor (Harvard Apparatus, Millis, MA, USA) connected to a Harvard student oscillograph.

4.2.2 | Biochemical determinations

Blood samples (obtained by cardiac puncture) from animals of both experimental groups were used for the determination of calcium. Calcium plasma levels were determined spectrophotometrically with commercial reagent kits (Wiener Laboratory, Rosario, Argentina).

4.2.3 | Tissue calcium analysis

Samples of the abdominal aorta and renal arteries were removed for the analysis of tissue calcium levels. Tissues were weighed and then heated to constant dry weight for 48 hours at 120°C. Dry tissue samples were dissolved in nitric acid (14 N) and left for 72 hours at room temperature. The samples were then centrifuged (2000 *g*, 10 minutes), and the supernatant removed. Strontium nitrate was added and calcium (μmol/g dry weight) measured by atomic absorption spectrophotometry.

4.2.4 | Pharmacokinetic studies

These studies were performed similarly to those described previously.[8,19,29] The animals were anaesthetized with sodium thiopental (70 mg/kg b.w., intraperitoneal [i.p.]). Both the femoral artery and the femoral vein were catheterized in order to obtain samples and to administer the test compound, respectively. A single bolus of Sulfanilamide (SA) (4.00 mg/kg b.w.) was administered (this dose does not saturate the transport systems; therefore, the kinetics can be adequately estimated).[8] Blood samples were collected at 0-15 minutes from the femoral artery for SA and acetylated-Sulfanilamide (ASA) assays. An equivalent volume of isotonic saline solution was then infused. Samples were frozen at −20°C until analysis. Determinations of SA and ASA concentrations were performed as described by Bratton and Marshall.[30]

4.2.5 | Determination of SA levels in renal and hepatic homogenates

Preparation of renal and hepatic homogenates

At the end of the pharmacokinetic studies, the kidneys and the liver were rapidly removed. Then, the renal and hepatic tissues

were cleaned, dried, weighed, and placed in isotonic saline solution. These tissues were thoroughly homogenized in 250 mmol/L sucrose, 10 mmol/L HEPES-Tris (pH 7.40) for SA and ASA analyses. Determinations of SA and ASA concentrations in tissues samples were performed as described by Bratton and Marshall.[30] The protein content was determined using the method of Sedmak and Grossberg.[31]

4.2.6 | Renal clearance studies

These studies were performed as previously described.[9-11] Briefly, the animals were anaesthetized with sodium thiopental (70 mg/kg b.w., i.p.). The femoral vein and femoral artery were catheterized and a bladder catheter (3 mm internal diameter) was inserted through a suprapubic incision. The animals were maintained in restraining cages throughout the experiment to facilitate collection of urine. A prime dose of SA (4.00 mg/kg b.w.) in 1 mL of saline solution was administered through the venous catheter. Then, a solution containing SA (6.00 g/L) and saline solution (9.00 g/L) was infused through the venous catheter employing a constant infusion pump (Pump 22; Harvard Apparatus, Holliston, MA, USA) at a rate of 1 mL/h per 100 g b.w. After equilibration for 45 minutes, urine was collected during two 20 minutes periods. Blood from the femoral artery was obtained at the midpoint of each clearance period. Arterial blood pressure was estimated throughout the experiments with a manometer inserted in the femoral artery. Determinations of plasma and urine SA and ASA concentrations were performed as described by Bratton and Marshall.[30] The renal clearance of SA and ASA were calculated by conventional formulae for each animal.

4.2.7 | Renal and hepatic blood flow determinations

Another set of experimental animals (control and treated rats) was used to evaluate renal and hepatic blood flow as previously described in our laboratory.[8,19]

Orange microspheres, 15 μm in diameter (Molecular Probes, Eugene, OR, USA), were infused as a bolus into the carotid artery (previously catheterized) followed by a washout with 1 mL saline. Arterial reference blood was collected from a catheter inserted in the femoral artery at a rate of 1 mL/min, which was continued for 60 seconds after injection. Five minutes after microsphere injection, kidneys and liver were removed. Blood samples were digested overnight with 89.2% KOH. Tissue samples were digested with 22.4% KOH for 24 hours. Digested samples were filtered using Poretics polycarbonate filters (Thomas Scientific, Swedesboro, NJ, USA). The fluorescence was measured at 540-560 nm. A known amount of yellow-green microspheres (505-515 nm) was added to each sample vial of blood and tissue prior to digestion. These microspheres acted as internal standards. Organ blood flow was calculated by the formula:

$$\text{Organ blood flow(mL/min)} = \text{fl}/\text{fl}_{\text{ref}} \cdot R\text{(mL/min)}$$

where fl is the fluorescence of the tissue, fl_{ref} is the fluorescence of the reference blood flow sample and R is the withdrawal rate of the reference blood flow sample.

4.2.8 | *N*-acetyltransferase activity assay

Preparation of renal and hepatic homogenates

Another set of experimental animals was used to evaluate *N*-acetyltransferase activity. The kidneys and the liver were rapidly removed; then, renal and hepatic tissues were cleaned, dried, weighed, and placed in isotonic saline solution. These tissues were thoroughly homogenized 30% P/V in 0.05 mol/L phosphate buffer (pH 6.80). Protein content was assayed using the method of Sedmak and Grossberg.[31]

4.2.9 | Enzyme activity determination

Ten microlitres of the above homogenate (dilution 1:4) was assayed according to methods firstly developed by Deguchi and Axelrod[32] and modified by Champney et al.[33] Each sample was incubated for 20 minutes at 37°C in the presence of 5 μL of 5.6 mmol/L tryptamine-HCl, 2 μL of 800 μmol/L acetyl-CoA, 2 μL of acetyl-(^{3}H)-coenzyme A (217 mCi/ mMoles; 40 nCi/ sample; Amersham Pharmacia Biotech, Little Chalfont, UK), and 1 μL of phosphate buffer, pH 6.80. The reaction was stopped by addition of 100 μL of 0.2 mol/L borate buffer, pH 10.00. The *N*-acetyl (^{3}H)-tryptamine formed was extracted by addition of 1 mL of chloroform with 5 minutes of continuous shaking followed by centrifugation for 30 seconds in a Beckman microcentrifuge. The aqueous phase was removed by aspiration. The organic phase was washed with 100 μL of 0.2 mol/L borate buffer, pH 10.00, shaken for 5 minutes, centrifuged, and the aqueous phase removed again. A 0.5 mL aliquot of the chloroform extract was allowed to evaporate to dryness in a glass scintillation vial. Three millilitres of Optiphase Hisafe 3 (Wallac Scintillation Products, Turku, Finland) was added prior to liquid scintillation spectrometry. Results were expressed as pmoles of *N*-acetyltryptamine formed per hour per milligram of protein.

4.2.10 | Statistical analysis

Statistical analysis was performed using an unpaired *t* test. When variances were not homogeneous a Welch's correction was employed. *P* values<.05 were considered significant. Values are expressed as mean±standard error (SE). For these analyses, GraphPad software (GraphPad Software, San Diego, CA, USA) was used. The concentration-time graphics for SA and ASA for each individual animal were fitted with the PKCALC computer program as previously described.[8,19,29,34,35] The choice of best fit was based on both the determination coefficient values (R^2) and *F*-test.

4.2.11 | Reagents

All chemicals were purchased from Sigma chemical (St Louis, MO, USA).

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DISCLOSURE

Authors declare no conflict of interest.

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